THE GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM THE METHANOLIC LEAF EXTRACT OF *Costuspictus*D.Don(COSTACEAE) FOR ENHANCING THE ORAL BIO-AVAILABILTY AND ITS ANTI DIABETIC ACTIVITY

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This is to certify that the dissertation entitled **"THE GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM THE METHANOLIC LEAF EXTRACT OF *Costus pictus* D.Don (COSTACEAE) FOR ENHANCING THE ORAL BIO-AVAILABILTY AND ITS ANTI DIABETIC ACTIVITY "** submitted by **Mrs. S. R. NANDHINI** in partial fulfilment of the requirement for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R. Medical University is a bonafied work done by her under my guidance during the academic year 2013-2014 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625020.

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STATION: Madurai.

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CONTENTS

INTRODUCTION

CHAPTER 1

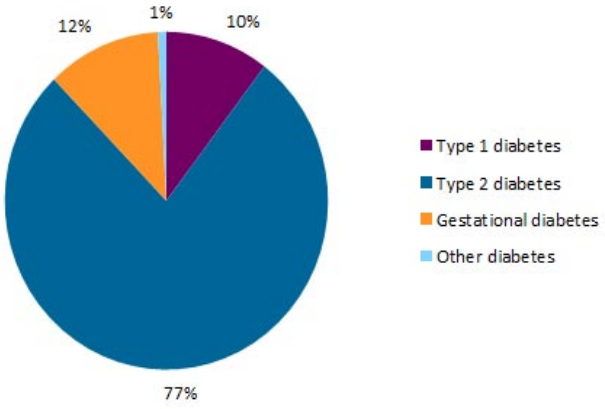
INTRODUCTION [1-23]

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Insulin is a hormone that regulates the blood sugar. Hyperglycemia, or raised blood sugar, is a common effect of uncontrolled diabetes and overtime leads to serious damage to different organs of the body, especially the eyes, kidneys, nerves, heart and blood vessels. Diabetes is one of the most prevalent diseases in the world. Diabetes has emerged as a major health care problem in India.

TYPES OF DIABETES

There are three types of diabetes namely Type 1 diabetes, Type II diabetes and Gestational diabetes. The **Type I diabetes** results when the body fails to produce insulin and hence requires the administration of insulin injection. The **Type II diabetes** results when the pancreas does not produce enough insulin or the cells not responding to insulin. Gestational diabetes results when the body of a pregnant woman does not secrete excess insulin required during pregnancy. The percentage of human beings affected by diabetes is shown in **Fig. 1.**

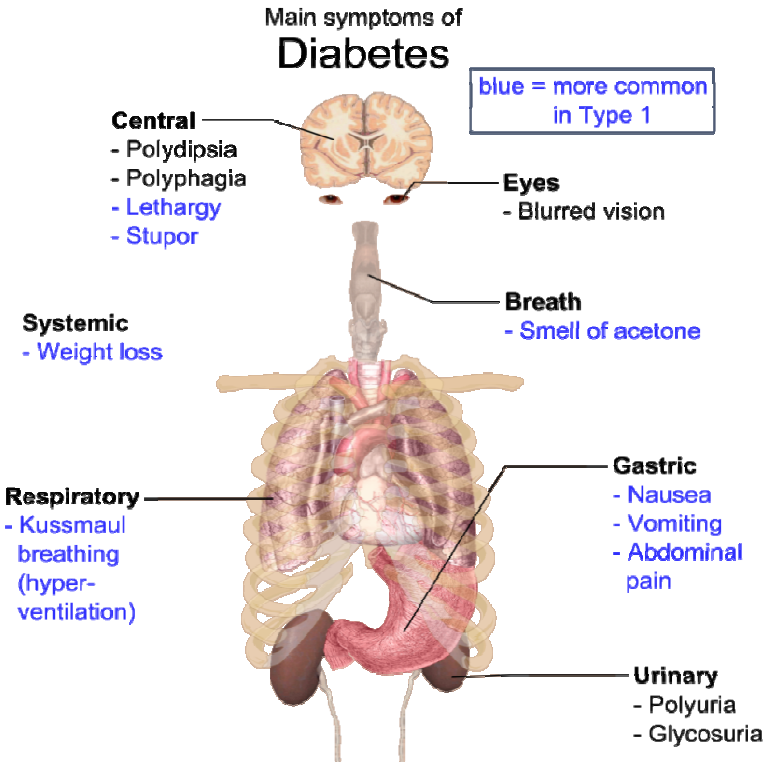
Fig. 1: Pie-chart representation of diabetes



SYMPTOMS OF DIABETES

The main symptoms of diabetes are polydipsia, polyphagia, lethargy, stupor.kassmaul breathing (hyper ventilation), weight loss, blurred vision, nausea, vomiting, abdominal pain, polyuria and glucosuria.[1] The various symptoms of diabetes are depicted in **Fig. 2.**

Fig. 2: Symptoms of diabetes



LONG TERM COMPLICATIONS OF DIABETES

Poorly controlled diabetes can results in damage of different organs and systems within the body. The major complications include cataract, glaucoma, retinopathy, neuropathy, damage of vagus nerves, nephropathy, hypertension, high cholesterol level, heart attack and stroke.[2,3]

'DIABETES CAPITAL' tag a burden on India's heart

Diabetes Atlas published by International Diabetes Federation (IDF), estimated that 40 million persons with diabetes in India in 2007 and this number is predicted to rise 70 million people by 2025.The countries with largest number of diabetic people will be India and China. World Health Organization has predicted that, India will be the world's diabetes capital by 2025.It is gradually becoming more dangerous than AIDS.There is an ample evidence to suggest that preventive measures to reduce the burden of diabetes are needed.[4,5]

ALLOPATHIC DRUGS USED IN DIABETES

As diabetes is a multifactorial disease leading to several complications, and therefore demands a multiple therapeutic approach. For example, to manage post-prandial hyperglycemia at digestive level, glucosidase inhibitors such as acarbose, miglitol and voglibose are used. These inhibit degradation of carbohydrates thereby reducing the glucose absorption by the cells. To enhance glucose uptake by peripheral cells biguanides such as metformin is used. Sulphonylureas like glibenclamide is insulinotropic and works as secretogogue for pancreatic cells.

Limitation of allopathic drugs

The limitations of allopathic drugs include taking the medicines throughout life time, high cost of medicines, side effects such as hypoglycemia, weight gain, gastrointestinal disturbances and liver toxicity etc. should be taking the medicine throughout the life.

HERBALS USED IN DIABETES[6]

Medicinal plants are being looked up for the treatment of diabetes. Many conventional drugs have been derived from prototypic molecules in medicinal plants. Metformin exemplifies an efficacious oral glucose-lowering agent. Its development was based on the use of *Galegaofficinalis* to treat diabetes. *Galegaofficinalis* is rich in guanidine, the

hypoglycemic component. Because guanidine is too toxic for clinical use, the alkyl biguanidessynthalin A and synthalin B were introduced as oral anti-diabetic agents in Europe in the 1920s but were discontinued after insulin became more widely available. However, experience with guanidine and biguanides prompted the development of metformin.

To date, over 400 traditional plant treatments for diabetes have been reported, although only a small number of these have received scientific and medical evaluation to assess their efficacy. Some of the common plants used for their antidiabetic potential are listed in **Table 1.** [7,8]

Table 1: Indian medicinal plants with antidiabetic and related beneficial effects

Plant Name	**Ayurvedic/common name/herbal formulation**	**Antidiabetic and other beneficial effects in traditional medicine**
Annonasquamosa	Sugar apple	Hypoglycemic and antihyperglycemic activities of ethanolic leaf-extract, Increased plasma insulin level
Artemisia pallens	Davana	Hypoglycemic, increases peripheral glucose utilization or inhibits glucose reabsorption
Areca catechu	Supari	Hypoglycemic
Beta vulgaris	Chukkander	Increases glucose tolerance in OGTT
Boerhaviadiffusa	Punarnava	Increase in hexokinase activity,decrease in glucose-6-phosphatase and fructose bis-phosphatase activity, increase plasma insulin level, antioxidant
Bombaxceiba	Semul	Hypoglycemic
Buteamonosperma	Palasa	Antihyperglycemic

Camellia sinensis	Tea	Anti-hyperglycemic activity, antioxidant
Capparisdeciduas	Karir or Pinju	Hypoglycemic, antioxidant, hypolipidaemic
Caesalpiniabonducella	Sagarghota, Fevernut	Hypoglycemic, insulin secretagogue, hypolipidemic
Cocciniaindica	Bimb or Kanturi	Hypoglycemic
Emblicaofficinalis	Amla, Dhatriphala, a constituent of herbal formulation, "Triphala"	Decreases lipid peroxidation, antioxidant, hypoglycemic
Eugenia uniflora	Pitanga	Hypoglycemic, inhibits lipase activity
Enicostemalittorale	Krimihrita	Increase hexokinase activity, Decrease glucose 6-phosphatase and fructose 1,6bisphosphatase activity. Dose dependent hypoglycemic activity
Ficusbengalenesis	Bur	Hypoglycemic, antioxidant
Gymnemasylvestre	Gudmar or Merasingi	Anti-hyperglycemic effect, hypolipidemic
Hemidesmusindicus	Anantamul	Anti snake venom activity, anti-inflammatory
Hibiscus rosa-sinesis	Gudhal or Jasson	Initiates insulin release from pancreatic beta cells
Ipomoea batatas	Sakkargand	Reduces insulin resistance
Momordicacymbalaria	Kadavanchi	Hypoglycemic, hypolipidemic
Murrayakoenigii	Curry patta	Hypoglycemic, increases glycogenesis and decreases gluconeogenesis and glycogenolysis
Musa sapientum	Banana	Antihyperglycemic, antioxidant
Phaseolus vulgaris	Hulga, white kidney bean	Hypoglycemic, hypolipidemic,

		inhibit alpha amylase activity, antioxidant. Altered level of insulin receptor and GLUT-4 mRNA in skeletal muscle
Punicagranatum	Anar	Antioxidant, anti-hyperglycemic effect
Salaciareticulata	Vairi	inhibitotory activity against sucrase, α-glucosidase inhibitor
Scopariadulcis	*Sweet broomweed*	Insulin-secretagogue activity, antihyperlipidemic, hypoglycemic, antioxidant
Swertiachirayita	Chirata	Stimulates insulin release from islets
Syzygiumalternifolium	Shahajire	Hypoglycemic and antihyperglycemic
Terminaliabelerica	Behada, a constituent of "Triphala"	Antibacterial, hypoglycemic
Terminaliachebula	Hirda	Antibacterial, hypoglycemic
Tinosporacrispa		Anti-hyperglycemic, stimulates insulin release from islets
Vincarosea	Sadabahar	Anti-hyperglycemic
Withaniasomnifera	Ashvagandha, winter cherry	Hypoglycemic, diuretic and hypocholesterolemic

Advantages of herbal medicines

The advantages of herbal medicines in treatment of diabetes include cheaper cost of medicines, available widely and less toxic and have fewer side effects as compared to allopathic drugs.All in all, diabetes can be prevented with herbal medicines and herbs are efficient and safe to one's health.

The hypoglycemic effect of some herbal extracts has been confirmed in human and animal models of type 2 diabetes. The World Health Organization Expert Committee on diabetes has recommended that traditional medicinal herbs be further investigated. Although several therapies are in use for treatment, there are certain limitations in allopathic drugs. Although several therapies are in use for treatment, there are certain limitations.Major hindrance in amalgamation of herbal medicine in modern medical practices is lack of scientific and clinical data proving their efficacy and safety. There is a need for conducting clinical research in herbal drugs, developing simple bioassays for biological standardization, pharmacological and toxicological evaluation, and developing various animal models for toxicity and safety evaluation.

INSULIN PLANT

*Costuspictus*D.Don is commonly known as spiral ginger, belonging to the family *Costaceae*. It is a magical cure of diabetes. Its leaf helps to build up insulin the human body. So, it is commonly known as Insulin plant in India. Insulin plant was grown in America and is becoming popular in India because of its medicinal value. It is now accepted and used widely as an Ayurvedic medicinal herb.

Consumption of the leaves is believed to lower blood sugar level. Diabetes patients are advised to chew two leaves per day in the morning and evening.After one week the patient should take one leaf in the morning and evening. This dosage should be continued for a month.It is turning out to be munching dietary supplement for diabetes. Allopathic doctors too recommend it and it is found to be effective in bringing blood sugar level under complete control [9-12]. Many *invivo* and *invitro* studies of this Insulin plant are carried out showed that this plant is having very potent antidiabetic effect. Though being widely used, no formulation containing this plant is available in the market. The patient feels difficulty in chewing the leaves for a month. In order to overcome this and to enhance the oral bio-availability and

pharmacological activity, protection from toxicity, physio-chemical degradation, improved tissue macrophages, to avoid repeated administration of the dose and to achieve the selective or targeted drug delivery towards a specific tissue or organ a attempt is made in this study to design and standardize a nano-formulation of *Costuspictus.*

INFLUENCE OF NANOPARTICLES IN HERBAL DRUGS

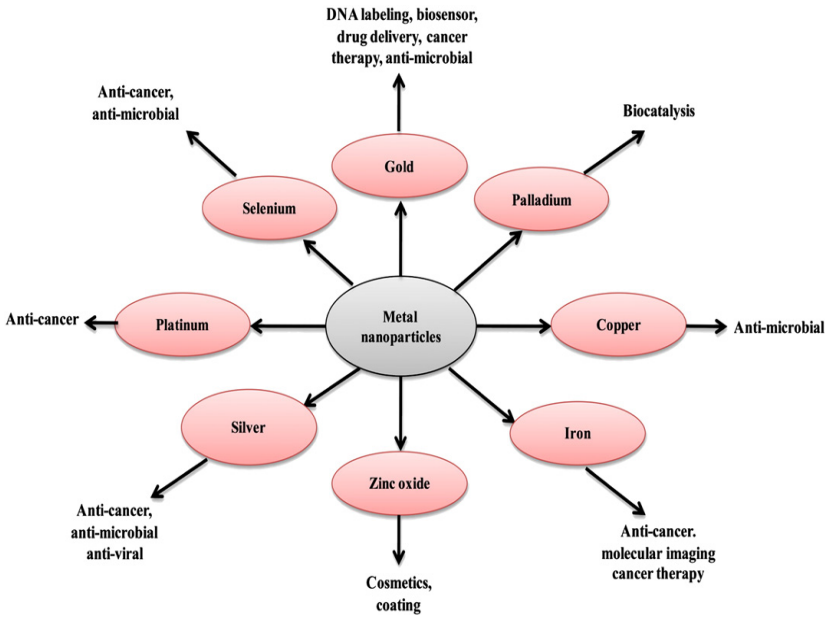
Herbal medicines have been widely used all over the world since ancient times and have been recognized by physicians and patients for their better therapeutic values as they have fewer adverse effects as compared to modern medicines. Phytotherapeutics needs a scientific approach to deliver the components in a sustained manner to increase patient compliance and avoid repeated administration. This can be achieved by designing Novel Drug Delivery System (NDDS) for herbal constituents. NDDS not only reduce the repeated administration to overcome non-compliance, but also helps to increase the therapeutic value by reducing toxicity and increasing the bio-availability. One such novel approach is nanotechnology. Nano-sized drug delivery system of herbal drugs has a potential future for enhancing the activity and overcoming the problems associated with plant medicines. Hence, integration of the nanocarriers as a NDDS in the traditional medicinal system is essential to conflict more chronic diseases like asthma, diabetes, cancer and others.[13-15]

GREEN SYNTHESIS OF SILVER NANOPARTICLES

Biosynthesis of nanoparticles is the active area of research for the last one decade. To date, metallic nano-particles are mostly prepared from nobel metals (ie Ag, Pt, Au and Pd), among the noble metals, silver (Ag) is the metal of choice in the field of biological system, living organisms and medicine[16-18]. Green synthesis of nanoparticles is an emerging branch of nanotechnology. Biosynthesis of nanoparticles using plant extracts is the favourite method of green, exploited to a vast extent because the plants are widely distributed, easily available,

safe to handle and with a range of metabolites and compatibility for pharmaceutical and biomedical applications as they do not use toxic chemicals in the synthesis protocols[19, 20]. Bio-inspired synthesis of nano-particles provides advancement over chemical and physical methods as it is a single step, cost effective and environment friendly and in this method there is no need to use high pressure, energy, temperature and toxic chemicals[21, 22, 23]. Types of metal nanoparticles and their applications in nanotechnology are depicted in the **Fig.3**

Fig.3: Types of metal nanoparticles and their applications in nanotechnology



It is a well-known fact that silver ions and silver-based compounds are highly toxic to microorganisms. This aspect of silver makes it an excellent choice for multiple roles in the medical field. Silver is generally used in the nitrate form to induce antimicrobial effect.

ADVANTAGES OF SILVER NANOPARTICLES:

- Increases the oral bio-availabilty.
- To overcome the poorly water soluble herbal medicines.
- It decreases the treatment dosage.
- Prolong action, rapid, single-step, eco-friend method.
- Non-toxic to the human body.

- Have instigated the researchers to investigate mechanism of metal ions uptake and bio-reduction by plants, and to understand the possible mechanism of metal nanoparticles formation in and by the plants.
- Green chemistry improves and/or protects our global environment.

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE [24-71]

The chapter provides retrieval of papers both from primary and secondary sources. The review of literature encompasses information on pharmacognosy, biological studies and phytochemistry of the various species of *Costus* and the green synthesis of silver nanoparticles.

Rathore AK *et al*., (1979) have isolated the steroids diosgenin, tigogenin, lanosterol and stigmasterol from the undifferentiated callus culture of *Costusspeciosus*. [24]

Iwu MM *et al.*, (1981) have a found that the rhizomes of *Costusafer* contain diosgenin (0.8%), stigmasterol (1.5%) and costugenin (3.0%) in addition to lanosteroltigogenin and β-sitosterol. The petroleum ether and chloroform extracts of the aerial parts yielded 0.12% and 0.16% sapogenin. [25]

Gupta MM *et al*., (1981) have isolated two new compounds from the roots of *Costusspeciosus* have been characterized as 24-hydroxy triacontan-27-one and 24-hydroxytriacontan-26-one by spectral data and chemical studies. Methyl triacontanoate, diosgenin and sitosterol have also been isolated and identified. [26]

Gupta MM *et al*., (1981) have isolated a new sterol from the roots of *Costusspeciosus* have been characterized as 5 α-stigmast-9(11)-en-3 (β – O) by spectral and chemical studies.[27]

Gupta M *et al*., (1982) have isolated two new compounds G and H from the methanolic leaf extract of roots of *Costusspeciosus* which have been characterized as 8-hydroxytriaconjtan-25-one ($C_{30}H_{60}O_2$, mp 95◦) and methyl tritriacontanote (C_{34} H_{68} O_2,mp 80-82°) respectively. [28]

Singh SB *et al.* , (1982) have isolated costusoside I ($C_{58}H_{96}O_{27}$, mp-220-24°) and costusoside J ($C_{57}H_{94}O_{27}.H_2O$, mp 248-50°) from the methanolic extract of the seeds of

Costusspeciosus and have been established the structure as 3-O-{β-D-glucopyranosyl (1→2)-α-L-rhamno-pyranosyl (1→2)-[α-L-rhamno-pyranosyl (1→4)]-β-D-glucopyranosyl-26-O-β-D-glucopyranosyl-22 α-methoxy-25R)-furost-5-en-3β, 26-diol and its 22-hydroxy compound respectively. [29]

Singh SB *et al*., (1982) have isolated steroidal saponins from seeds of *Costusspeciosus* and have been elucidated as β–sitosterol-β-D-glucopyranoside, prosapogenin-β of dioscin, prosapogenin–A of dioscin, gracillin, 3-O[α-L-rhamnopyranosyl ((1→2) β-D-glucopyranosyl], 26-O-[β-D-glucopyranosyl]-22 α-methoxy-(25R)-furost-5-en-3β, 26diol, methyl protodioscin: and protodiosin. [30]

Iwu MM (1982)have done a survey of phytotherapeutic profile of Nigeria herbs. 32 plants are used by the tribes in Nigeria for anti-inflammatory and arthritis. Among these, 3 plants viz., *Costusafer, Lonchocarpuscyanescens and Terminaliaivorensis* were selected for clinical trials and pharmacological test. The pharmacological test was conducted using different solvent of the herbs, reduced carrageenin- induced oedema of the rat paw, and checked diarrhoea induced by arachidonic acid, castor oil and ameliorated all signs associated with adjuvant- induced polyarthritis in rats.[31]

Suri RK *et al*., (1986) have isolated six fatty acids viz., palmitic (55.97%), oleic (22.75%), linoleic (6.78%), diosgenin (1.91%) and 3 sugars, glucose, galactose and rhamnose from the seeds of *Costusspeciosus*. [32]

Lin RC *et al*., (1996) have isolated a new steroidal saponin, aferoside A from the roots of *Costusafer*. Its structure was eatablished as 3-0-{(*β*-D-apiofuranosyl- (1 to 2)}-{*α*-L-rhamno pyranosyl (1 to 4) β-D−glucopyranosyl)}-25-(R) spirost-5-en-3β-ol by chemical transformation and various spectroscopic methods, mainly 2D NMR techniques (COSY, HMQC and HMBC). [33]

Lin RC *et al*., (1997) have isolated two new steroidal saponins, aferosides B (1) and C (2), together with the known saponins, dioscin (3) and paraphyllin C (4) from the roots of *Costusafer*. Kaemperol 3-O-α-L-rhamno pyranoside was obtained from the aerial parts of the plant. The structure of the new compounds was elucidated principally by 2D NMR spectral methods. A structural revision of the sugar sequence was made the previously reported saponinaferoside A (6), saponin (1-4) and 6 did not show any ability to potentiate *in vitro*cisplatin cytotoxicity in a human colon cancer lines. [34]

Souccar C *et al.*, (1997) have evaluated the antiuroliatic activity of *Costusspicatus* (0.5g/kg/day) did not alter the body weight, but reduced the weight of both formed calculi and urinary bladder stones respectively, 65% and 48% of control. Treatment of rats with the extract of either *Cerropiaglazious* or*Cupheabalsamona* at doses that reduced the blood pressure (0.5g/kg/day) did not affect the animal weight nor did it influence the growth of urinary bladder stones. The results showed the possible antiurolithiatic activity of the extract of *Costusspicatus* confirming folkore information. [35]

Aguiyi*et al.*, (1998) have reported that the aqueous extract of *Costusafer* leaf and stem showed pronounced antibacterial activity against tested micro organisms. The observation supports its use in traditional medicine for the treatment of cough and sores. [36]

Pereira B *et al*., (1999) have isolated a new furostanol glycoside from the rhizomes of *Costusspicatus*. Its structure was established as (3β, 22α, 25R)-26-(β-D glucopyranosyloxy)-22-methoxyfurost-5-en-3-yl-O-D-apio-β-D-furanosyl-(1→2)-O-[6-deoxy-α-1-mannopyranosyl-(1→4)]β-D-glucopyranoside. The structure was identified using ^{1}H &$^{13}C^{NMR}$ spectra including 2D NMR spectroscopic technique (COSY, HETCOR & COLOC) and chemical conversions. [37]

Viel TA *et al*., (1999) have evaluated the antiurolithiatic activity of the aqueous extract of *Costusspiralis* Roscoe in rats. On oral treatment with the aqueous extract (0.25 and

0.5g/kg/day) after four weeks surgery reduced the growth of calculi on implants of calcium oxalate crystals or zinc disc in the urinary bladder of rats. It did not prevent hypertrophy of the organ smooth musculature. The results indicated that the aqueous extract of *Costusspiralis* was endowed with antiurolithiatic activity thus confirming the folklore claim. [38]

Da Silva *et al*., (1999) have isolated a new steroidal saponin from the rhizomes of *Costusspicatus* and its structure was elucidated as ((3β, 22α, 25R)–26-(β–D-glucopyranosyloxy)-2-5-en-3-yl-O-D-apio-β-D-furanosyl-(1→4)-O-[α-L-rhamnopyranosyl-(1→2)] -β-D-glucopyranoside by means of IR, MS, NMR and chemical evidence.[39]

Da Silva *et al*., (2000) have isolated a new flavanoldiglycoside, 3,5-dihydroxy-7,4'-dimethoxy flavone 3-O-neohesperidoside, together with four known flavonol 3-O-neohesperidosides from the leaves of *Costusspiralis* and their structures were elucidated by a combination of spectroscopic methods and chemical reactions. [40]

Da Silva *et al*., (2000) have isolated two flavonoldiglycosides, tamarixetin 3-O-neohesperidoside, kaempferide 3-O-neohesperidoside and the known quercetin-3-O-neohesperideside together with six other known flavonoids from the leaves of *Costusspicatus* and their structures were elucidated by a combination of spectroscopic and chemical methods. The flavanoldiglycosides were evaluated for inhibitory activity of nitric oxide production by activated macrophages. [41]

Indrayanto G *et al*., (2001) have isolated 3-O-[beta- D-glucopyranosyl- (1"→2')-β-D-glucopyranosyl], 27- O-β-D-glucopyranosyl-(25R)-spirost-5-ene-3β, 27-diol from the cell suspension cultures of *Costusspeciosus*, following incubation with diosgenin, and its structure was elucidated using a combination of one and two dimension ^{1}H and ^{13}C NMR spectral data, positive and negative ions ESMS spectral data. [42]

Anaga*et al.*, (2004) have studied the methanolic leaf extract of *Costusafer* Ker. for pharmacological activities *in vitro and in vivo*. The methanolic leaf extract showed moderate local anaesthsetic property, about twice less than lignocaine of the same concentration, on guinea pig wheal test. The extract exhibited a biphasic antihyperglycemic activity. At 200mg/kg body weight, p.o, it decreased the blood glucose level by 50% in streptozocin induced hyperglycemia in male rats in 60mins post dosing. However, doses above 200mg/kg body weight. p.o caused increase in blood glucose level, potentiating the action of streptozocin. At 10µg/mL the extract induced about 98% glucose uptake in differentiated 3T3–L1 adipocytes when compared with insulin (340nm). [43]

Behera L.M *et al*., (2007) have done a survey of some useful medicinal plants against gynaecological disorders by the tribes of Ramkhol village forest of Bara Pahad hill range in Bargarh district (Orissa). Traditional plants used for the treatment were listed out including *Costusspeciosus*. [44]

George A *et al*., (2007) have done the phytochemical analysis of Insulin plant (*Costuspictus*). 18 chemical compounds were identified in the leaves of the plant by using GC-MS. From the chromatogram, it was evident that the major component in the ether fraction is bis (2'- ethyl hexyl)-1, 2-benzene dicarboxylate (59.04%). The major component in the acid fraction are haxadecanoic acid (44.53%) and 4,8,12,16- tetramethylheptadecan 4-olide (27.86%). [45]

Jayasri M A *et al*., (2008) have reported the anti oxidant activity of leaves and rhizome of *Costuspictus* in methanol, aqueous, ethanol and ethyl acetate are assessed using different models like DPPH, β-carotene, Deoxy ribose, Super oxide anion, reducing powers and metal chelating assay at different concentrations. Total phenolic content showed good anti oxidant activity of about 89.5% and 90.0% when compared with standard BHT (85%) at

a concentration of 400mcg/ml. Results obtained revealed that methanolic extracts of both leaves and rhizomes possess higher anti oxidant activity than other extracts. [46]

Kosalge SB *et al*., (2008) have investigated the anthelmintic activity of the aqueous extract of *Cassia fistula* bark (Leguminoseae) (Bahava) and*Costusspeciosus* rhizomes (Zingeberaceae) (Pewda) using earth worms *Eiseniafoetida* and tapeworm *Taeniasaginata* as test animals. Various concentrations (10-50mg/mL) of plant extracts were tested. Both the aqueous extracts showed vermicidal activity and found to be effective as anthelmintic. [47]

Sharma V *et al*., (2009) have demonstrated the anti-cancer potential of the root extract of *Costusspeciosus*. The extract of the root part were screened for *in vitro* cytotoxicity by means of SRB assay on five human cancer cell lines; colon cancer cells (COLO-205, HT-29, SW-620), liver cancer cells(HEP-2) and prostrate cancer cells (DU-145). All the extracts of the plant showed remarkable cytotoxicity effect on each human cancer cell line in the range of 70-92%. [48]

Rajesh *et al*., (2009) have studied the anti diabetic activity of various extracts of *Costusspeciosus* rhizomes. The anti hyperglycemic activity of petroleum ether, chloroform, methanolic and aqueous extracts was evaluated on overnight fasted, streptozotocin(STZ) induced diabetic rats. Blood sugar level (BGL) monitored at 0, 30, 60, 12 and 240min suggested that all the extracts resulted in reduction of BGL significantly except pet ether extract. Aqueous and methanolic extracts reduced initial BGL of 387 to 120mg/dL and 303 to 161mg/dL respectively at the end of 240min. [49]

Qari SHM *et al*., (2009) have studied the genotoxic and anti-genotoxic potential of aqueous extract of the rhizomes of*Costusspeciosus* using the comet asayswith three concentrations (0.50, 10.0 and 20.0 mg/mL). The human lymphocyte cultures were treated for 24h with different concentrations to test for genotoxicity as a single treatment as compared to control. The anti-genotoxic effects of the extract were tested in human

lymphocyte cultures exposed to the DNA damage inducing agent ethyl methane sulfonate (EMS), either alone or combined with the different concentrations of the extract, which was added to the cultures before, simultaneously with or after the EMS. The results demonstrated that the rhizomes of *Costusspeciosus* aqueous extract is not genotoxic culture human lymphocytes and indicate that when added to lymphocyte cultures with or before the EMS it has anti-genotoxic activity against EMS-induced DNA damage. [50]

RaoValluru J and Mani NS (2009) have carried out the ethnobotanical survey of medicinal plants used for the treatment of menstrual problems in SalurMandal of Vizianagaram district, A.P, India. Among the 15 plant species, *Aristolochiaindica* (Aristolochiaceae), *Costusspeciosus* (Costaceae) were most commonly used plant species for the treatment of menstural disorder by the tribes.**[51]**

Chakraborthy GS *et al*., (2009) have studied the antibacterial and antifungal activity of *Costusspeciosus* petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extracts (Zingiberaceae). The different extracts showed remarkable inhibitory action against various gram positive and gram negative bacterial and two fungal species. [52]

Shubhaand Anusuya D *et al*., (2010) have reported the anti oxidant and nutrient content of micro propagated *Costuspictus* inoculated with beneficial soil micro organisms. The nutrient status of plants inoculated with *Glomusfasiculatum, Azetobacter, Aspergillusawamori, Frateuriaaurentia and Trichodermaviride*showed appreciable increase in total antioxidant content and also enhances the nutrient value of plants. [53]

Reddy JL *et al*., (2010) have studied the antibacterial activity of the leaf essential oil of *Costuspictus* against ten bacterial strains by agar well diffusion method. The leaf oil exhibited pronounced activity against all the tested micro organisms. [54]

Quintans LJ *et al*., (2010) have studied the antinociceptive and anti inflammatory effect of*Costusspicatus* in experimental animals by using acetic acid, formalin, hot plate test

while inflammation was induced by carrageenan. Following oral administration of methonolic extract of *C. spicatus* (100, 200 and 400mg/kg) significantly reduced the number of writhes (52.8, 43.1 and 55.3%) respectively in the writhing test and the number of paw licks during phase I (61.9, 54.1 and 92.1%) and phase II (62.5, 82.9 and 98.1%, all doses) during the formalin test to the control group animals. The reaction time during hot plate was significantly increased and was dose-dependent whereas, pretreatment with nalaxone rigorously reduced the analgesic potential of methonolic extract of *C. spicatus,* which suggested that participation of opioid system in the modulation of pain induced by methonolic extract of *C. spicatus*. The results unlikely provoked to the motor abnormality, as methonolic extract of *C. spicatus*- treated mice did not show any performance alteration during the Rota-rod test. The administration of 200-400mg/kg (i.p) of methonolic extract of *C. spicatus* shows an anti-inflammatory effect during the carragenan test; this was based on inference with inflammatory mediator synthesis. The results indicated that the methonolic extract of *C.spicatus* shows significant effect of antinociceptive and anti inflammatory actions. [55]

Britto, R.M *et al*., (2011) have studied the arterial contractility and investigate its M.O.A from the aqueous fraction of *Costusspiralis*(Jacq.) Roscoe leaf. The aqueous fraction effect on the cardiac contractility was studied on isolated electrically driven guinea pig left atria. Atropine and tetraethyl ammonium (TEA) were employed to mechanism of aqueous fraction. The role of calcium in this effect was also studied by analyzing the aqueous fraction effect on the Bowditchth's phenomenon, as well as by studying whether it could interfere with the concentration effect curve for calcium chloride, isopreterenol and BAY K 8644. They proposed that the aqueous fraction of *Costusspiralis* leaf depresses myocardial contractility by reducing the L-type calcium current and by decreasing the intra cellular calcium transient.[56]

Mohamed S *et al*., (2011) have carried out antituberculosis screening using tetrazolinium bromide microplate assay (TEMA) method. The MIC was in the range of 1000 to 400µg/mL for 38 plant extracts tested. Among these, the methanolic leaf extract of *Angiopteris erector* exhibited the highest activity with MIC of 400µg/mL. Five other extracts, namely *Costusspeciosus* (stem and flowers) and *Piper sarmentosum* (whole plant), *Plucheaindica*(flower), and *Tabernaemontanacoronaria* (leaf) exhibited antituberculosis activity, each with MIC of 800µg/mL.[57]

Nadumane VK *et al*., (2011) have studied the anti proliferative and apoptotic effect of *Costuspictus* D. Don aqueous and alcoholic extracts on fibrosarcoma HT-1080 cell lines. Different concentrations of these extracts were evaluated for their cytotoxicity by tryptophan blue dye exclusion method and 3-(4,5-dimethyl thiazol-2yl)- 2,5- diphenyltetrazolinium bromide (MTT) assay on cancer cell lines (Fibrosarcoma HT-1080) and a normal cell line(human peripheral lymphocyte). The apoptotic potential was analyzed by DNA fragmentation analysis of the treated cells. The ethanol extract was found to be anti-proliferative and cytotoxic at lower concentrations and induced cell death in HT-1080 fibrosarcoma cells. The ethanol extract of *C. pictus*was found to be anti-proliferative and cytotoxic at lower concentrations and induced cell death in HT-1080 fibrosarcoma cells. The ethanol extract at the same concentrations had no cytotoxicity on normal lymphocytes. Compared to ethanol extracts, aqueous and methanol extracts were less effective. [58]

Green synthesis of Silver nanoparticles

A review of literature on silver nanoparticles of various plants and extracts are presented in order to evaluate whether the nanparticles are useful in the reducing the doses etc.

Jain D *et al*., (2009) have described the green synthesis of silver nanoparticles from 1mM silver nitratesolution through the extract of papaya fruit as reducing as well as capping

agent. Nanoparticles were characterized using UV-visible absorption spectroscopy, FTIR, XRD and SEM. X-ray diffraction and SEM analysis showed the average particle size of 15nm as well revealed their cubic structure. Biologically synthesized silver nanoparticles were found to be highly toxic against different multi drug resistant human pathogens. [59]

Christensen L *et al*., (2011) have synthesized silver nanoparticles using *Murrayakoenigii*leaf extract and the reported the effect of broth concentration in reduction mechanism and particle size. The rapid reduction of silver ions was monitored using UV-visible spectrophotometry and showed the formation of silver nanoparticles within 15minutes. Transmission Electron Microscopy and Atomic Force Microscopy analysis showed the synthesis of silver nanoparticles is varied from 10-25nm and have the spherical shape and the X-Ray Diffraction analysis confirms the nanocrystalline phase of silver with FCC crystal structure. It was found that the increasing broth concentration increases the rate of reduction and decreases the particle size. [60]

Manopriya M *et al*., (2011) have reported the synthesis of silver nanoparticles using the leaf extracts of *Euphorbia hirta*and *Neriumindicum*. Synthesized particles are characterized by UV – Spectrophotometer, SEM, FTIR and X- ray diffraction analysis. The Debye-Scherrer equation was used to calculate particle sizes and the average size of the silver nanoparticles synthesized by *E.hirta*was 31nm and by *N.indicum*was 29nm. Further the synthesized silver nanoparticles were tested against common bacterial pathogens.[61]

Ponaruselvam S *et al*., (2012) have developed a novel approach for the green synthesis of silver nanoparticles using aqueous leaves of *Cantharan*thus*roseus* (Linn. G. Don) The scanning electron microscopy showed the formation of silver nanoparticles with an average size of 35-55nm. X-Ray Diffraction analysis showed that the particles were crystalline in nature with the face centered cubic structure of bulk silver nanoparticle with the

broad peaks at 32.4, 46.4 and 28.0 respectively. The obtained silver nanoparticles showed the antiplasmodial activity against *Plasmodium falciparum*. [62]

Firdhouse JM *et al*., (2012) have synthesized the silver nanoparticles using the ethanolic leaves of *Pisoniagrandis* (R.Br) using three different methods and the completion of the reaction was monitored with UV-visible spectrophotometer and characterized using XRD, Scherrer's equation and SEM analysis. The particle size was found to be less than 150nm, spherical shape as confirmed from SEM and XRD analysis.[63]

Rajan K *et al*., (2012) have described the synthesis of gold nanoparticles from the methanolic leaf extract of *Toona ciliate bark.* The synthesized gold nanoparticles showed a surface Plasmon band around 55nm when analysed via UV –Visible Spectroscopy, indicated the gold nanoparticles of nano dimension (10^{-9}mm). Transmission Electron Microscopy study of gold nanoparticles revealed that the particles are spherical, poly dispersed nanoparticles of varying sizes ranging from 40-75nm along the encapsulating cage. [64]

Akl M *et al*., (2012) have synthesized the silver nanoparticles (AgNPs) of mulberry leaves extract at room temperature. Silver nanoparticles were characterized using UV-visible absorption spectroscopy, scanning electron microscopy (SEM) and X-ray diffraction (XRD). Further, silver nanoparticles showed effective antibacterial activity toward *Staphylococcus aureus*and *Shigella*sp. [65]

Pavani KV *et al*., (2013) have synthesized the silver nanoparticles using methanolic extract of flowers of *Ipomoea indica*. The formation of silver nanoparticles was confirmed by Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD) studies. SEM image revealed that silver nanoparticles are quite polydispersed, the size ranging from 10nm to 50nm. The formation of crystalline silver nanoparticles was confirmed using X-ray diffraction analysis. [66]

Roy K *et al*., (2013) have demonstrated the synthesis of silver nanoparticles by reducing silver nitrate with fruit extract of grape (*Vitisvinifera*). Characterization of the metallic nanoparticles was done by UV- Vis Spectroscopy, Dynamic Light Scattering (DLS) and Energy Dispersive X-ray Spectroscopy (EDX). The particle size and lattice image of the silver nanoparticles was studied by Transmission Electron Microscopy (TEM). The antibacterial activity of these nanoparticles was studied against *Bacillus subtilis*and *Escherichia coli*. Growth curves of bacteria in presence of silver nanoparticles showed inhibition of growth suggesting antibacterial property of the nanoparticles. [67]

Iravani S *et al*., (2013) have studied the production of silver nanoparticles using *Pinuseldarica*bark extract and optimization of the biosynthesis process. The effects of quantity of extract, substrate concentration, temperature, and pH on the formation of silver nanoparticles are studied. TEM images showed that biosynthesized silver nanoparticles (approximately in the range of 10–40 nm) were predominantly spherical in shape. The preparation of nanostructured silver particles using *P. eldarica*bark extract provides an environmentally friendly option, as compared to currently available chemical and/or physical methods.[68]

Sulaiman GM *et al*., (2013) have synthesized the silver nanopaticles from leaves extract of*Eucalyptus chapmaniana* and test the antimicrobial of the nanoparticles against different pathogenic bacteria, yeast and its toxicity against human promyelocyticleukemia (HL-60) cell line. The synthesized silver nanoparticles exhibited a maximum absorption peaks at 413 nm due to characteristic surface plasmon resonance. X-Ray Diffraction analysis showed that the particles were crystalline in nature with the face centered cubic structure of bulk silver nanoparticle with the broad peaks at 38.50° and 44.76° respectively. The synthesized silver nanoparticles efficiently inhibited various pathogenic microorganisms and reduced viability of the (HL-60) dose dependent manner. [69]

Sivaraman D *et al*., (2013) have investigated the synthesis and characterization of Silver nanoparticles (AgNPs), and their antimicrobial effect on gram positive and gram negative bacteria by using hydro alcoholic leaf extract of *Ipomoea aquatica*Forsk (HAEIA). The bio-reduced silver nanoparticles were appropriately characterized by using UV-Vis spectroscopy, particle size distribution and zeta potential. The particle size of bio synthesized silver nanoparticles is found to be in the range of 77-624 nm with polydspersity index (PDI) of 0.207 clearly indicates that the particles are in very narrow size distribution. The zeta potential value of 6.16 mV showed that the nanoparticles prepared are stable.[70]

Yadav SK*et al*., (2013) havebiosynthesized the silver nanoparticles using different solvents of *Terminaliachebula*Retz.fruit bodies. They exhibited a maximum absorption peaks ranging between 410-430nm due to characteristic surface Plasmon resonance. The spherical shaped silver nanoparticles were observed. The antibacterial property of synthesized nanoparticles was observed by Agar Well Diffusion method against different clinically isolated multi-drug resistant gram positive and gram negative bacteria.[71]

The review of literature has shown that *Costus*species have potent antidiabetic activity and no formulations of the same have been formulated and evaluated. Hence an attempt in this dissertation has been made to synthesize silver nanoparticles of the methanolic extract of the *Costuspictus* D. Don and evaluate the same for antidiabetic activity.

AIM AND SCOPE

CHAPTER 3

AIM AND SCOPE [72-76]

Nanotechnology is one of the most active areas of research in modern material science. The rapid development of nanotechnology has opened the possibility of controlling and manipulating structures at the molecular level and led to the creation of novel surface architectures and materials. Traditional biomedical applications incorporate the use of nanotechnology in a broad spectrum of areas. [72]

The growing of environmental friendly nanoparticles, researchers are using green methods for the various metal nanoparticles. Now-a-days, biosynthesis of nanoparticles by plant extracts is currently under exploitation. The development of biologically inspired experimental processes for the synthesis of nanoparticles is evolved in to an important branch of nanotechnology.[73]

Nanocrystalline silver nanoparticles have found tremendous applications in the field of high sensitivity biomolecular detection and diagnostics, antimicrobials and therapeutics. Several nano-oriented approaches are being intended in order to optimize the technological aspect of the drugs. The use of these processes has dramatically enhances the dissolution rates *in vitro* and bioavailability *in vivo* of many drugs.[74]

An attempt is made to enhance the dissolution rates and hence bio-availability and furthermore to decrease the treatment dosage of the *Costuspictus*D. Don extract by preparing its silver nanoparticles. The present study is designed to evaluate and compare the effects of the methanolic leaf extract of *Costuspictus*D. Don and its silver nanoparticles.

The ethno pharmacological studies revealed that the leaves of *Costus pictus*D. Don are having potent anti diabetic activity, diuretic activity, hepatoprotective activity. It is also

used to treat rashes, asthma, and bronchitis, reduce fever and to eliminate intestinal worms. The plant also possesses analgesic and anti-inflammatory, anti-urolithiatic activity. The infusion of this plant is used to treat renal disorder.The leaf essential oil is having antibacterial activity. The crude ethanolic extract of this plant possess antiproliferative and apoptotic effects. The phytochemical studies of *Costus pictus*D. Don have reported the presence of proteins, carbohydrates, alkaloids, flavonoids, phenolic compounds, tannins, saponins. [75, 76]

Based on the above ethnomedical information and studies available, the present research work has been framed to carry out the following studies on the leaves of *Costus pictus* D. Don.

1. **Pharmacognostical studies**
    - ❖ Macroscopic and microscopic evaluation.
    - ❖ Standardization parameters
    - ❖ Quantitative analytical parameters
    - ❖ Powder microscopy and Fluorescence analysis of powder and extracts.
2. **Phytochemical studies**
    - ❖ Preliminary phytochemical screening
    - ❖ Quantitative estimation of secondary metabolites
    - ❖ TLC & HPTLC fingerprint analysis
3. **Synthesis of methanolic leaf extract of *Costus pictus* D. Don silver nanoparticles (MECPAgNP'S)**
4. **Characterization of methanolic leaf extract of *Costus pictus* D. Don silver nanoparticles (MECPAgNP'S)**
    - ❖ Determination of particle size, polydispersity Index & zeta potential.
    - ❖ UV-Visible spectroscopical analysis

- ❖ Morphology of MECPAgNP's by SEM analysis

5. **Pharmacological studies**

i) In vitro antioxidant activity by

- ❖ Scavenging of 2,2-Diphenyl -1-picrylhydrazyl (DPPH)
- ❖ Scavenging of Hydrogen peroxide
- ❖ Total Antioxidant activity by Phosphomolydenum method
- ❖ Ferric Reducing Antioxidant Power Assay
- ❖ Reducing power assay

ii) *In vitro* antidiabetic activity by

- ❖ Inhibition of alpha amylase enzyme assay
- ❖ Inhibition of alpha glucosidase enzyme assay
- ❖ Glucose uptake in yeast cells
- ❖ Non – enzymatic glycosylation of haemoglobin assay

PLANT PROFILE

CHAPTER 4

PLANT PROFILE [9-12, 77-79]

BIOLOGICAL SOURCE	**:** *Costuspictus*D. Don
FAMILY	**:** Costaceae
SYNONYMS	**:***Costusheiroglyphica; Costusmexicanus Costuscongestus*

SYSTEMATIC POSITION

Rank	: Species
Domain	: Eukaryota (unranked)
Kingdom	: Plantae
Phyllum	: Magnoliophyta
Class	: Liliopsida
Order	: Zingeberaceae
Family	: Costaceae
Genus	: *Costus*
Species	: *pictus*

VERNACULAR NAMES

English	: Painted spiral ginger; Stepladder ginger; Spotted spiral ginger; Insulin plant
Hindi	: Keukand
Tamil	: Kottam
Malayalam	: Insulin chedi.
Marathi	: Pushkarmula
Sanskrit	: Kemuka
Gujarati	: Pakarmula

DESCRIPTION

Costuspictus is an ornamental plant of Mexico. Itis a relatively new entrant to Kerala. It is a perennial herbaceous plant grows up to 3m in height. It is called as Insulin plant because it builds up insulin in the human body. The Catch phrase of this plant is **"A Leaf a day keeps Diabetes away".**

GEOGRAPHICAL DISTRIBUTION AND OCCURRENCE

*Costuspictus*D. Don is distributed along the coast from Mexico to Costa Rica,Beliza, Nicarauga, Gautemala, Hondurasand Elsavador. It is locally known as Cana brava or Cana de jabali in Mexico.

HABIT AND HABITAT

*Costuspictus*D. Don is a perennial evergreen herb. It is well grown in part shade to part sun, but well grown in full sunlight with adequate water. It will grow in containers and in the ground. It requires acidic and neutral soil PH and the soil should be sandy, loamy and clay.

ETHNOMEDICINAL INFORMATIONS FOR WHOLE PLANT:

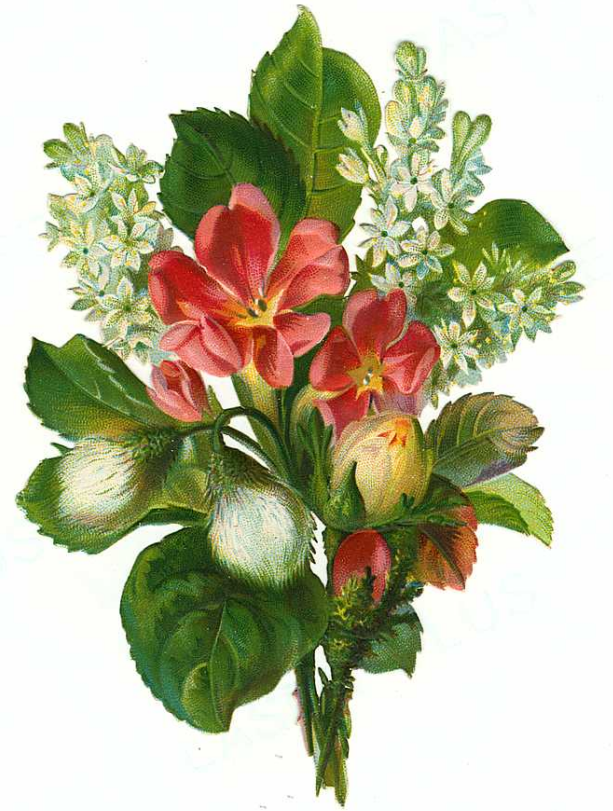
(1) In Traditional Medicine it is used to promote longevity, treat rashes, asthma, and bronchitis, and reduce feverand to eliminate intestinal worms.

(2) The leaves are reported to have a potent anti-diabetic activity. The plant also possesses analgesic and anti-inflammatory, anti-urolithiatic, diuretic, hepatoprotective activity.

(3) The infusion of this plant is used to treat renal disorder.

(4) The leaf essential oil is having antibacterial activity.

(5) The crude ethanolic extract of this plant possess antiproliferative and apoptotic effects.



PHARMACOGNOSTICAL EVALUATION

Fig.4.HERBARIUM of *Costuspictus* D. Don

CHAPTER - 5

PHARMACOGNOSTICAL EVALUATION[80-91]

Pharmacognostical study is the preliminary step in the standardization of crude drugs. The detailed pharmacognostical evaluation gives valuable information regarding the morphology, microscopical and physical characteristics of crude drugs. Pharmacognostic studies have been done on many important drugs, and the resulting observations have been incorporated in various pharmacopoeias. The pharmacognostic study gives the scientific information regarding the purity and quality of crude drugs. [80]

MATERIALS AND METHODS

Collection of specimens

The plant specimens were collected from Dr.Gour, Rock Garden, G. Amsapuram, Theni District, Tamil Nadu, India during the month of August 2013. The plant was identified and authenticated by Dr. L. Stephen, Lecturer in Botany, American College. The authenticated herbarium sheet has been placed at the Dept. of Pharmacognosy, College of Pharmacy, Madurai Medical College. A copy of the herbarium has been presented in **Fig.4** special care was taken to select healthy plants and normal organs for macroscopical studies. The macroscopical features of the plant are presented in **Figs. 5.1 to 5.6.**

SECTION A - MACROSCOPICAL EVALUATION

Morphology: Some of these gross morphological characters of drugs such as shape, size, margin, apex, venation, are identification features of drugs. These features give valuable information about the drugs.

Cellmorphology: It includesthe study of morphological characteristics of particular cells.The various aerial parts of *Costuspictus* D. Doncollected for macroscopical evaluation

were leaves, flower and cone. The photographic representation of the macroscopic features of the plant is shown in **Figs. 5.1 to 5.6.**

SECTION B - SENSORY EVALUATION [81]

The term sensory evaluation refers to organoleptic evaluation. Characters such as color, odor, taste, texture, touch etc. are evaluated with the help of sense organs. The sensory characteristics of organized as well as unorganized drugs give an idea about the quality, identity, purity of drugs.

SECTION C - MICROSCOPICAL EVALUATION

Microscopical study of organized crude drugs is an important parameter for evaluation. Another important aspect of microscopical evaluation is the study of surface constants. These evaluations allow more detailed examination of the phytodrug to identify the organized drug by its histological character. It provides detailed information about the crude drugs by virtue of its property to magnify the fine structures of minute objects to be visualized and thereby confirm the structural details of the plant drugs under evaluation. It can also be used in the determination of the optical as well as micro chemical properties of the crude drug.

The samples of the leaves were cut and removed from the plant and fixed in FAA (formalin-5mL + acetic acid-5mL + 70% ethyl alcohol-90mL). After 24h of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol as per the schedule given by Sass, 1940. [82] The infiltration of the specimens was carried by gradual addition of paraffin wax (m.p. 58-60°C) until TBA solution attained super saturation. The specimens were then cast into paraffin blocks.

Sectioning

The paraffin embedded specimen was sectioned with the help of rotary microtome. The thickness of the sections was 10-12µm, de-waxing of the sections was carried out by customary procedure (Johansen, 1940). [83] The sections were then stained with toludine blue as per the method published by O'Brien *et al.* (1964). [84]Toludine blue, a polychromatic stain was used for staining as the results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. The sections were also stained with saffranin and fast-green and iodine wherever necessary.

Leaf clearing

Two methods were used for studying the stomatal morphology, venation pattern and trichome distribution. Paraffin embedded leaf was used for para-dermal sections. From these sections, the epidermal layers as well as vein islets were studied. Another method employed was clearing leaf fragments by immersing the material in alcohol (to remove chlorophyll) followed by treating with 5% sodium hydroxide. The material was rendered transparent due to loss of cell contents. Epidermal peeling by partial maceration employing Jeffrey's maceration was also done. Glycerin mounted temporary preparations were made for cleared materials.

For study of elements of xylem, small fragments of leaves were macerated with Jeffery's maceration fluid.

Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell component were studied and measured.

Photomicrographs

The photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used and for the study of starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized they appear bright against dark background. The magnifications of the anatomical features are indicated by the scale-bars in the photographs.

The microscopic features of the leaves was presented in **(Figs.6.1 to 10.2)**

SECTION D - QUANTITATIVE ANALYTICAL MICROSCOPY

Quantitative analytical microscopy is useful for the measurement of cell contents of the crude drug and thus helps in their identification, characterization and standardization. After several numbers of determinations, a clear idea about the identity and characteristic features of the drug can be obtained. This method is based on the comparison between the characteristics observed in the sample and those standard materials of known origin.

Determination of Leaf Constants

The stomatal number, stomatal index, vein islet number, vein termination number were determined on fresh leaves using standard procedures.

Determination of Stomatal number and Stomatal Index[88]

It is a very specific criterion for identification and characterization of leafy crude drugs. Four different types of stomata are often available for matured leaves that are distinguished by their form and arrangement in the surrounding cells.To study the stomatal number and stomatal index of leaf, the leaf was subjected to epidermal peeling.

Stomatal number

Stomatal number is the average number of stomata per square mm of epidermis of the leaf.[91]

Procedure

A leaf of 5x5mm^2 was taken in a test tube containing chloral hydrate solution and heated on a water bath for about 15min, until the fragments are transparent. The upper and lower epidermis was peeled out separately by using a forceps. The transparent leaf was placed on the slide and mounted with few drops of glycerine,cover slip was placed on the top. With the help of camera lucida and stage micrometer 1mm square was drawn on the black chart. The epidermis and the stomata were traced. The number of stomata present in the area of 1mm square including the cell if at least half of its area lies within the square was counted. The results for each field were calculated and the average number of stomata per square mm was measured and their values are tabulated in **Table 2.**

Stomatal Index [89, 90]

Stomatal index is the percentage which the number of stomata to the total number of epidermal cells, each stoma being counted as one cell.Stomatal index was calculated by using the following formula - S.I = S/(E+S) x 100; where S.I is thestomatal index, S is the number of stomata in 1sq mm of the leaf and E is the number of epidermal cells in the same unit area.

Procedure

For stomatal index, the glycerine mounted leaf peeling was taken and circle (O) like mark for each stomata and cross (X) like mark for each epidermal cell was marked on the chart paper. From these values, stomatal index was calculated from the formula and the values are tabulated in **Table 2.**

Determination of vein islets and vein terminations[87,89]

Vein Islets

Vein islet is used to indicate the minute area of the photosynthetic tissues encircled by the ultimate divisions of the vascular strands. The area of the leaf that is preferably taken is

from the lamina midway between the midrib and margin.The number of vein islet/sq. mm. is termed as vein islet number. This number per unit area of leaf is constant.

Vein Terminations

An ultimate free end or termination of a vein islet is called vein termination. The number of vein terminals/ sq. mm. of the leaf surface is termed as vein termination number.

Procedure [90]

A piece of fresh leaf was cut from the middle portion of the lamina avoiding midrib and the margin and taken in a test tube and boiled with chloral hydrate solution in a water bath, until they are cleared enough for observation. The cleared fragments were stained with saffarin solution and a temporary mount was prepared with glycerol solution. The stage micrometer was placed and examined under 10 x objective and an area of 1sq. mm. was drawn. The cleared leaf piece was placed on the microscope stage, the vein islets and terminals within the square were counted. The results obtained for each number of vein islets and terminals in 1 sq.mm.are tabulated in **Table 2.**

SECTION E - POWDER ANALYSIS AND POWDER MICROSCOPY

Powder Microscopy

The dried leaf material was powdered and the powder was passed through sieve no. 60 for the study of powder microscopy. Chloral hydrate, glycerine, iodine, phloroglucinol, hydrochloric acid (1:1), lacto phenol etc. were employed as mounting medium. The pictorial representation was presented in **Fig.11.**

Powder analysis

The reaction of the powder sample with different chemical reagents was carried out as per Kay (1938) and Johanson (1940). The results are presented in **Table 3.**

Fluorescence analysis

The fluorescence analysis of the drug powder as well as the plant extracts of*Costuspictus* D. Don were carried out by using the method of Chase and Pratt (1949). The results are presented in **Table 4.**

SECTION F - STANDARDIZATION PARAMETERS

The evaluation of ash values, loss on drying, foreign organic matter and extractive values etc., gives a clear idea about the specific characteristics of crude drug under examination, besides its macro-morphology or cyto-morphological, microscopical nature in both its entire and powder form. These diagnostic features enable the analyst to know the nature and characteristic of crude drugs. The procedures recommended in Indian Pharmacopoeia 1996, WHO guidelines 1998 and The Ayurvedic Pharmacopoeia of India were followed to calculate the total ash, water-soluble ash, acid-insoluble ash and loss on drying. The percentage of extractive values for different solvents was also determined.[85,86]

Determination of Volatile Oil

Volatile oils are the odorous principals found in various plant parts. Because they evaporate when exposed to air at ordinary temperatures, they are called volatile oils, ethereal oils or essential oils. Volatile oils are mainly composed of mono and sesquiterpenes and phenolic compounds.[87]

Volatile oils are extracted by

i) Steam distillation

ii) Solvent extraction

iii) Mechanical means such as eculle and enfluerage technique [88]

Procedure

The essential oil was isolated from*Costuspictus*by hydro distillation method. An accurately weighed 100g of the fresh leaves was crushed and introduced in to the round

bottom flask with a mixture of water and glycerine. The graduated receiver was filled with the water to avoid air bubbles. A few porcelain pieces were added. Then the distillation was allowed to continue for 3h. After 3h, heating was stopped and the oil obtained was collected in the volatile oil container. The percentage yield of the oil was calculated using the following formula - [Volatile oil obtained/ wt. of the sample taken] x 100. The oil content of the plant material was calculated in mL/100g of plant material and the result was reported in the **Table 5.**

Determination of Moisture Content (Loss on drying)

Loss on ignition is the loss in weight in percentage w/w resulting from a part of any test material that is volatilized and driven off under specified conditions. The test is performed on finely powdered material; lumps, if any should be broken up with the aid of a mortar and pestle.[85]

Procedure

An accurately weighed 10g, of the drug sample in a petridishwhich was previously dried under the conditions specified in IP'96. The powder was distributed evenly by gentle sidewise shaking. Then the petridish was dried at 105^{o}C for 5h and weighed. After 5h, the petridish was cooled in the desiccator to room temperature. The drying and weighing was continued at one hour interval until difference between two successive weighing corresponds to not more than 0.25%. The L.O.D was calculated with reference to the amount of air dried powder. The results obtained are presented in **Table 5.**

Determination of Ash value[85,86]

Ash content

The residue remaining after incineration is the ash content of crude drugis the residue of the extraneous matter (eg. sand and soil) that adhere to the plant surface.Ash content is determined by three different methods; which measures

a) Total ash

b) Acid insoluble ash

c) Water soluble ash

Determination of Total ash

An accurately weighed 2g, of the powdered drug was taken in a tared crucibleand incinerated by gradually increasing the heat to 500-600^{o}C until it was free from carbon. Then, it was cooled and weighed for constant weight. The percentage yield of ash with reference to the air dried drug was calculated.The results obtained are presented in **Table 5.**

Determination of Acid- insoluble ash

Acid insoluble ash is the residue obtained after boiled the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

Method

To the total ash obtained 25mL of 2M hydrochloric acid was added and boiled for 5min. Then, the insoluble matter was collected in an ashless filter paper and washed with hot water until the filtrate became neutral and ignited for 15min at a temperature not exceeding 450^{o}C and cooled in a desiccator and weighed. The percentage yield of acid-insoluble ash with reference to the air dried drug was calculated. The results obtained are presented in **Table 5.**

Determination of Water soluble ash

Water soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water.

Method

The total ash obtained was mixed with 25mL of water and boiled for 5min in a water bath. The insoluble matter was collected in the ashless filter paper and washed with hot water

and ignited in a crucible for 15min at a temperature not exceeding 450°C and cooled in a desiccator and weighed. The weight of this residue in mg was subtracted from from the weight of total ash. The percentage of water soluble solution ash with reference to the air dried drug was calculated. The results obtained are presented in **Table 5.**

Determination of Foreign Organic Matter [86]

Any organ or part of organ other than those specified in the definition and description is termed as foreign organic matter.

Procedure

An accurately weighed 100g of the plant material and spread it out in a thin layer. The plant material was detected by inspection with unaided eye or by the use of a lens (6x). Then, the foreign matter was separated and weighed. The percentage yield of the foreign organic matter was calculated with reference to the drug taken. The results obtained are presented in **Table 5.**

Determination of Extractive Values

Extractive values are used to determine the amount of active constituents in a given amount of medicinal plant material, when extracted with suitable solvent. The extraction of crude drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of drug and solvent used. The use of single solvent can be means of providing preliminary informationon the quality of the particular drug sample.[85,86]

Determination of ethanol soluble extractive value[86,87]

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of ethanol in a conical flask for 24h,shaking frequently during the first 6h and allowed to stand for 18h. Thereafter, filter rapidly taking precautions against loss of ethanol. 25mL, of the filtrate was evaporated to dryness in a china dish and dried at 105°C and

weighed. The percentage of ethanol soluble extract with reference to the air dried drug was calculated. The results obtained are presented in **Table 5.**

Determination ofmethanol soluble extractive value

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of methanol in a conical flask for 24h,shaking frequently during the first 6h and allowed to stand for 18h. Thereafter, filter rapidly taking precautions against loss of methanol. 25mL, of the filtrate was evaporated to dryness in a china dish and dried at 105^{o}C and weighed. The percentage of methanol soluble extract with reference to the air dried drug was calculated. The results obtained are presented in **Table 5.**

Determination of Chloroform soluble Extractive Value

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of chloroform in a conical flask for 24h,shaking frequently during the first 6h and allowed to stand for 18h. Thereafter, filter rapidly taking precautions against loss of chloroform. 25mL, of the filtrate was evaporated to dryness in a china dish and dried at 105^{o}C and weighed. The percentage of chloroform soluble extract with reference to the air dried drug was calculated. The results obtained are presented in **Table 5.**

Determination of water soluble extractive value

The procedure adopted under ethanol soluble extractive was followed using water as a solvent. The results obtained are presented in **Table 5.**

Determination of petroleum ether soluble extractive value

The procedure adopted under ethanol soluble extractive was followed using petroleum ether as a solvent. The results obtained are presented in **Table 5.**

Determination of benzene soluble extractive value

The procedure adopted under ethanol soluble extractive was followed using benzene as a solvent. The results obtained are presented in **Table 5.**

Determination of ethyl acetate soluble extractive value

The procedure adopted under ethanol soluble extractive was followed using benzene as a solvent. The results obtained are presented in **Table 5.**

Determination of Foaming Index[86,87]

Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The saponins are high molecular weight containing phytoconstituents having detergent activity. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of foaming index.The WHO has recommended the following procedure for the determination of the foaming index.

Method

An accurately weighed 1g of a coarsely powdered drug sample was taken in 500mL of conical flask containing 100mL of boiling water. Then, the conical flask was maintained at moderate boiling for 30min. Then, the flask was cooled and filtered in to a 100ml volumetric flask and sufficient amount of distilled water was added to dilute to volume. The decoction obtained was poured in to 10 stoppered test tubes in successive portion of 1mL, 2mL, 3mL, etc., up to 10mL and the volume in each test tube was adjusted to 10mL with water. Then, the test tubes were stoppered and shaken them in a length wise motion for 15sec(two shakes/sec) and allowed to stand for 15min and the height of the foam was measured.The results are assessed as follows;

If the height of the foam in every test tube was less than 1cm, the foaming index was less than 100.

If the height of the foam of 1cm was measured in any test tube, the volume of the plant material decoction in this tube (a) was used to determine the index.If the height of the foam was more than 1cm in every tube, the foaming index was over 1000. In this case repeat the determination using a new series of dilutions of the decoction in order to obtain a result.

The foaming index was calculated using the formula - 1000/a; where a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm was observed. The result obtained is presented in **Table 5.**

Determination of Swelling Index[86]

Swelling index is the volume in mL taken up by the swelling of plant material under specified conditions. The medicinal plant materials like gums, mucilage, and pectin have swelling property.

Method

An accurately weighed 1g of the powdered drug material was taken in the 25mL glass stoppered measuring cylinder. 25mL of water was added and shaken the mixture thoroughly every 10min for 1h. Then, allowed to stand for 3h at room temperature. The volume in mL occupied by the plant material was measured, including sticky mucilage was observed.The result obtained is presented in **Table 5.**

RESULTS AND DISCUSSION

SECTION A - MACROSCOPICAL OBSERVATION

*Costuspictus*D. Don is a perennial herb. The plant is a hardy vigorous growing ginger. The stem is red with spiral light leaves and airy, the tissue paper like flowers. **(Fig. 5.1)**

Leaf

The leaves are simple, large fresh looking spirally arranged, oblong-lonceolate being dark green above and lighter green below. Acumin has filiform point 2-3mm. The shape of the leaf is narrowly elliptic with the length 10 to 25cm and width 2.5 to 6cm. **(Fig. 5.3)**

Flower

The flowers are in terminal cone, **(Fig. 5.4)**yellow in colour with an orange red tip and lasts for 3-4 days and spread 1.5-2m. The flowers do not produce an aroma. It flowers throughout spring and summer. **(Fig. 5.5)**

Bract: The length and width of the bract is 2-4cm and 2-4cm respectively. The bract is green with maroon outer margin.

Bracteolate : The length of the bracteolate is 1.6-2.1cm.

Calyx : The calyx is 4-10mm long with lobes 2-3.5 mm long. The length of calyx is 6.5 cm.

Corolla : The corolla is yellow to red in colour and 65mm long.

Labellum : The lobes are yellow to red striped and irregularly trilobulate and the mid lobule is tridenate. The shape is obovate spread, margin crenulate.

Stamen : The anther is cream or yellow and 7-8mm long. The apex is dark red and 4 to 4.5cm long and 0.9 to 1.5cm wide.

Stem

Fig.5.1: Diagram showing Macroscopy of *Costus pictus* D. Don

Fig. 5.2: HABITAT OF *Costus pictus* D. Don

Fig. 5.3: WHOLE PLANT OF *Costuspictus* D. Don

Fig. 5.4: CONE OF *Costuspictus* D. Don

Fig. 5.5: FLOWERS OF *Costus pictus* D. Don

Fig.5.6. LEAF DORSAL AND VENTRAL VIEW OF *Costus pictus* D. Don

The red painted stem enhances the beauty of glossy linear leaves and strongly spirally canes. **(Fig. 5.3)**

Fruits

The fruits are globose capsules containing obovoid seeds.

SECTION B – SENSORY EVALUATION

Nature - Coarse Powder

Colour - Pale Green

Odour - Aromatic odour

Taste - Sour

SECTION C – MICROSCOPICAL EVALUATION

T.S. of Leaf through adaxially depressed Midrib

Leaf is shallowly boat shaped, having a group of vascular bundles. This part of the leaf represents the midrib. **(Fig.6.1)**The midrib is 750µm thick. It consists of thin epidermal layer of small squarish cells on the adaxial side and fairly thick cylindrical epidermal cells on the adaxial side **(Fig. 6.1).** The ground tissue includes, wide, thin walled compact parenchyma cells with shrunken walls.**(Fig. 6.2)**

The vascular system of the midrib includes an abaxial band of three vascular bundles and adaxial single median bundle. The adaxial vascular bundles have only a few xylem elements; these are two or three wide circular xylem elements, but the phloem is a large mass of sieve elements.**(Fig. 6.2)**

Fig.6.1: T.S. of leaf through adaxially depressed midrib

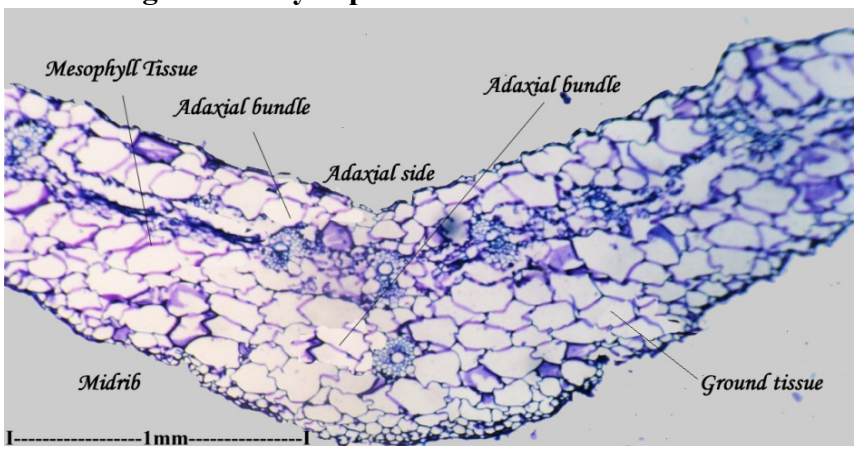


Fig. 6.2: Midrib vascular bundle enlarged

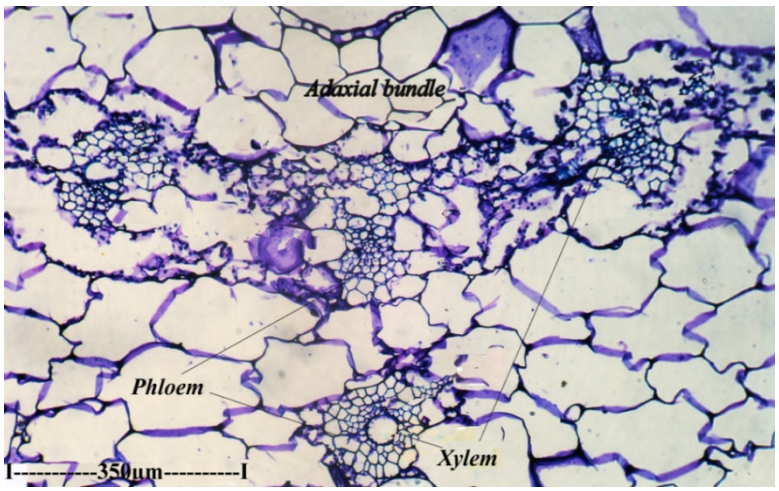
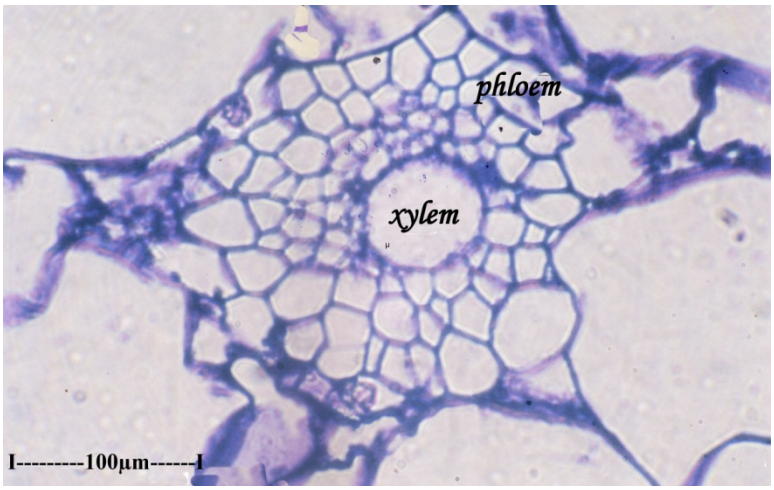


Fig. 6.3: The adaxial median bundle enlarged



The abaxial solitary bundle has a single, wide, circular thin walled xylem elements and a small clusters of phloem elements located on the upper side **(Fig.6.3).** The vascular bundle is surrounded by wide layer of parenchymatous bundle sheath.

The vascular bundles of the lamina occur in a single horizontal row of the median part of the lamina. The lamina- bundles also have one or two wide circular xylem elements and thick clusters of phloem and parenchymatous bundles heath **(Fig.7.1 & 7.2).** The adaxial epidermal layers of the lamina consist of thick, cylindrical cells.

The abaxial epidermis is thin and it consists of narrow tubular cells. The adaxial epidermis is apostomatic. The adaxial epidermis is stomatiferous. **(Fig.7.1)**

Crystal distribution (Fig.8.1 & 8.2)

Calcium oxalate crystals of minute particle are aggregated in to large masses in the leaf mesophyll cells. These microcrystal masses are random and diffuse in distribution. The crystals are located in ordinary unspecialized cells.

Epidermal cells and stomatal morphology (Fig.9.1 & 9.2)

The epidermal cells and stomata were observed in the paradermalsectious**(Fig.9.1 & 9.2).** The epidermal cells are rectangular or polygonal; the anticlinal wallsare thick and straight. The stomata are diffuse in distribution. They are surrounded by the two pairs of lateral subsidiary cells. This type of stomata is called hexacytic type. The guard cells are 15 X 30µm in size.

Venation pattern (Fig.10.1 & 10.2)

The leaf exhibits fairly prominent parallel main veins. The main parallel veins are interconnected by thin, less prominent horizontal veins.

Fig.7.1: T.S. of lamina with laminar vascular bundle

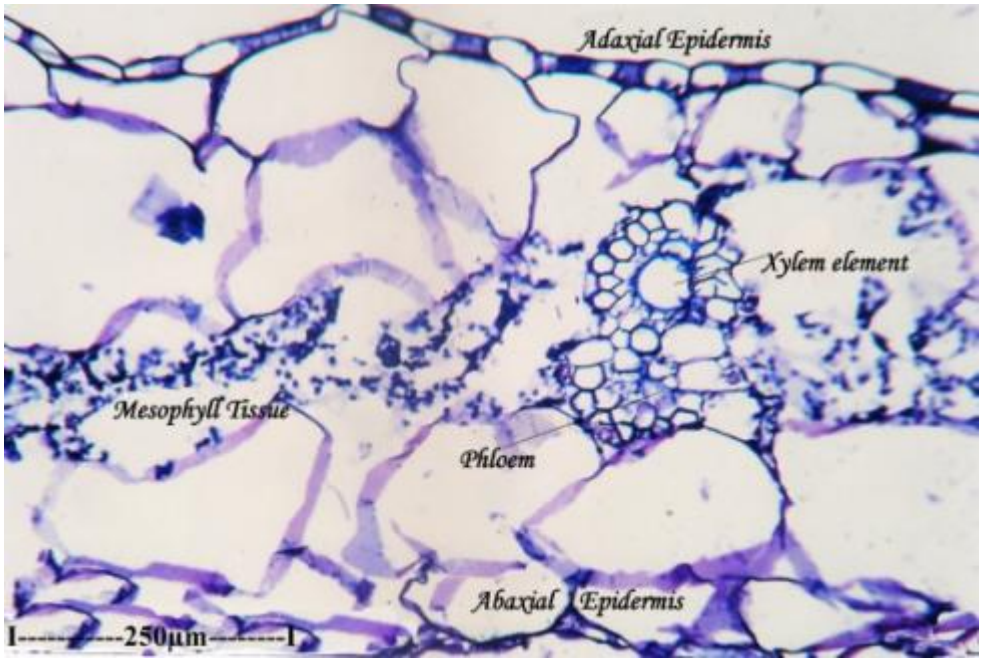


Fig.7.2: Lateral laminar vascular enlarged

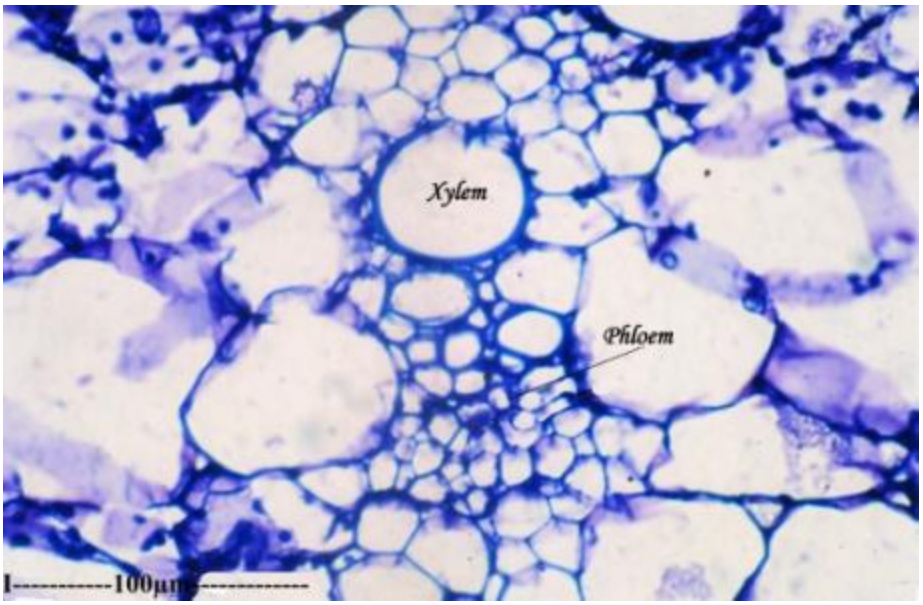


Fig. 8.1: Crystal distribution in the leaf mesophyll

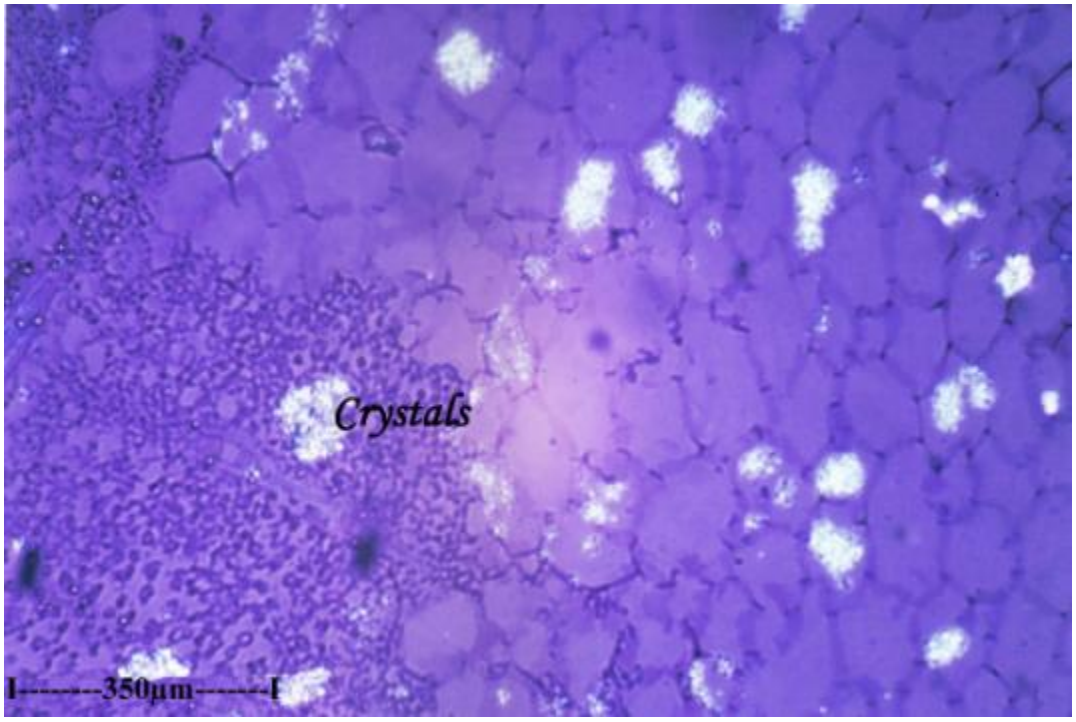


Fig. 8.2: One crystal enlarged

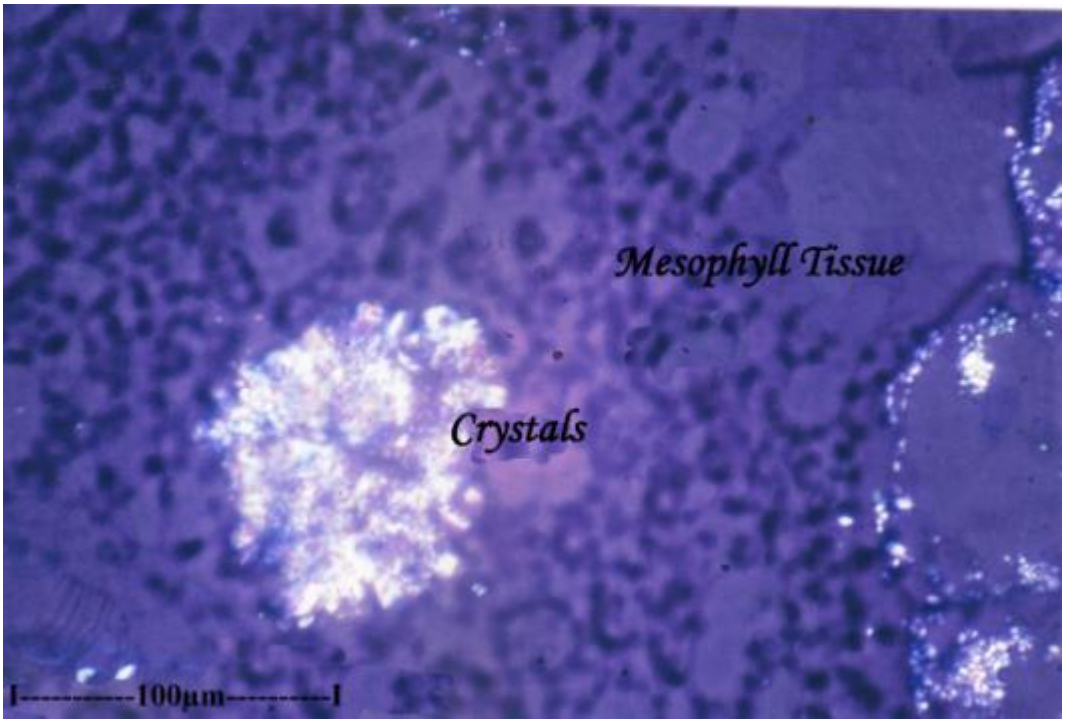


Fig.9.1: Paradermal section showing stomata on the abaxial epidermis

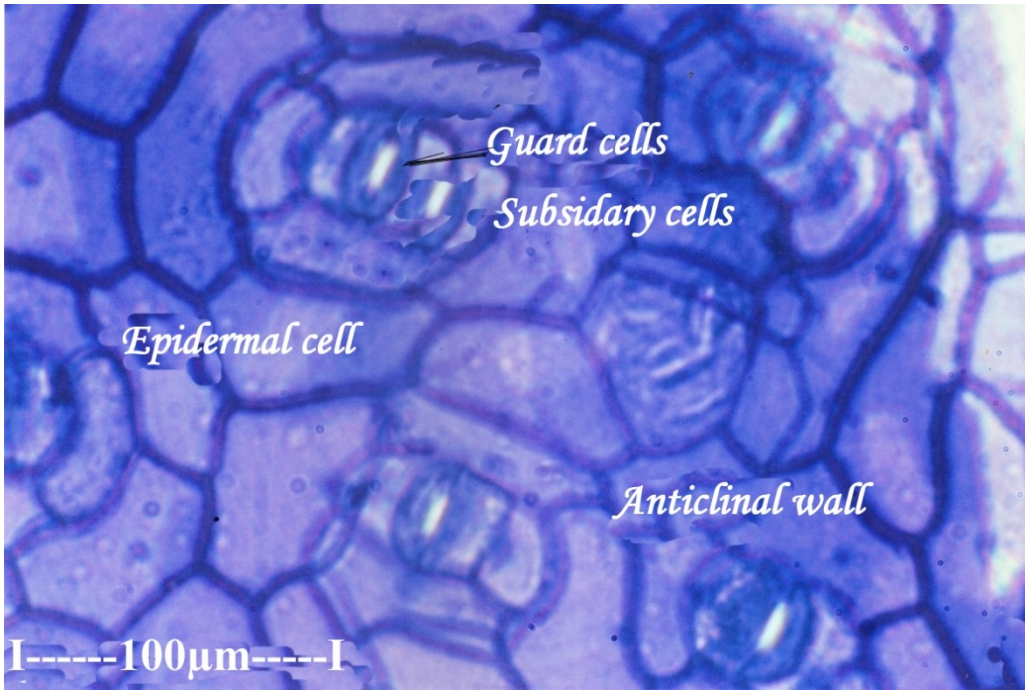


Fig. 9.2: One stoma enlarged

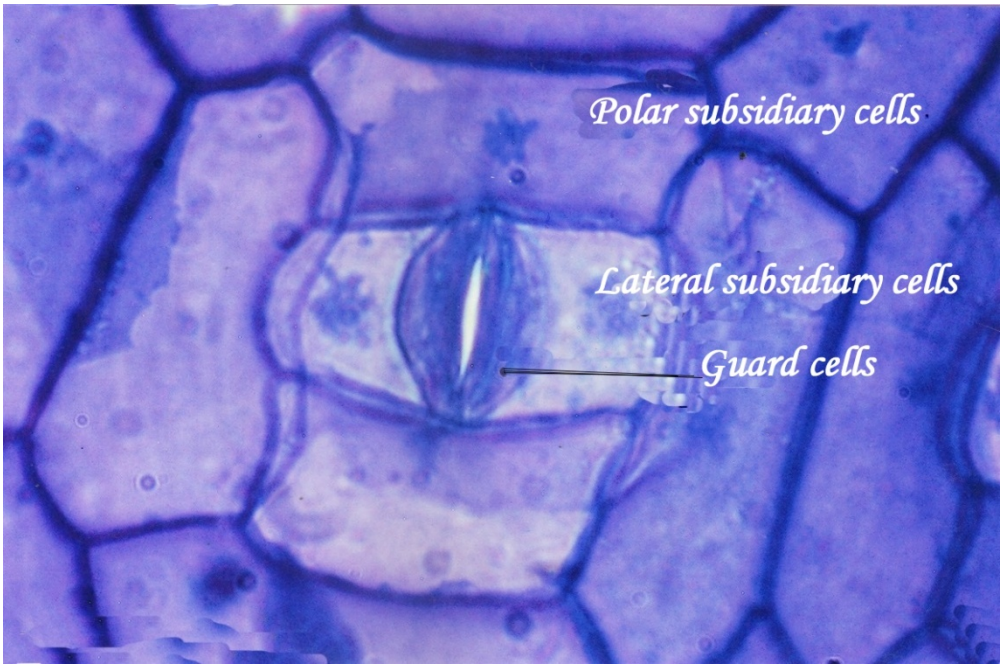


Fig. 10.1: Venation pattern of the lamina

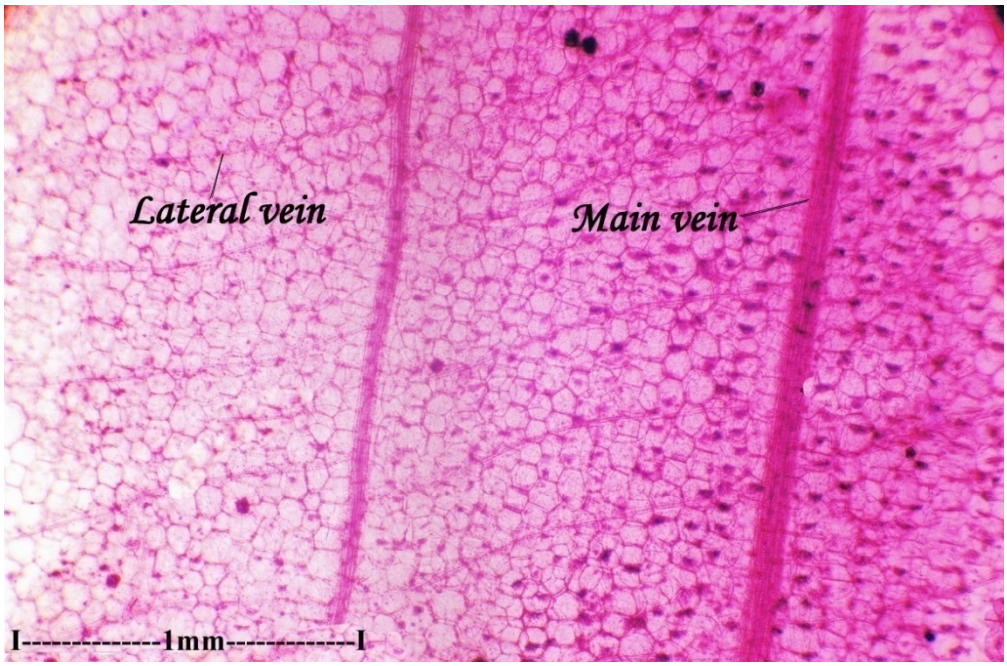
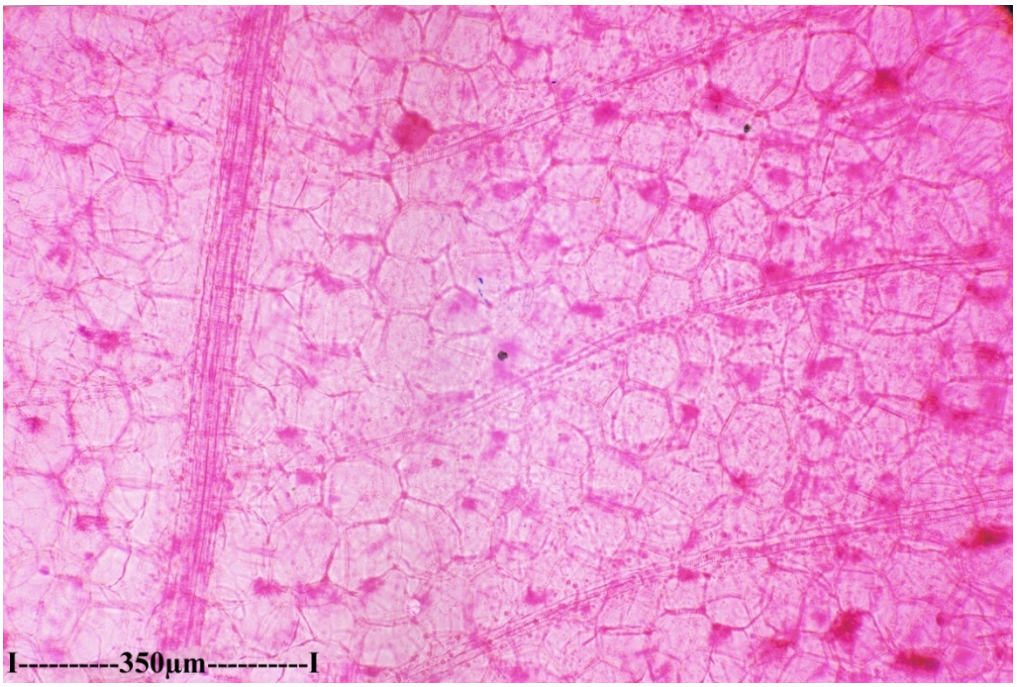


Fig. 10.2: Venation pattern of the lamina enlarged



SECTION D - QUANTITIATIVE ANALYTICAL MICROSCOPY

The results obtained for the determination of leaf constants are presented in **Table 2.** From the table, it can be observed that the number of stomata in the upper epidermis was found to be 2.17±0.05 while in the lower epidermis it was 13.0±0.28.The stomatal index in the upper epidermis and lower epidermis was 3.5±0.28 and 10.38±0.23 respectively. The vein islet number was found to be 4.1±0.057 and the vein termination number was 3.9±0.088. These values help in identification of leaf of *Costuspictus* D. Don from other species of the genus *Costus.* Since these values are unique for each plant.

Table 2: Quantitative analytical microscopical parameters of the leaf of *Costuspictus*D.Don

S. No.	Parameters*	Values obtained
1	Stomatal number in upper epidermis	2.17±0.05
2.	Stomatal number in lower epidermis	13.0±0.28
3.	Stomatal index in upper epidermis	3.5±0.28
4.	Stomatal index in lower epidermis	10.38±0.23
5.	Vein islet number	4.1±0.057
6	Vein termination number	3.9±0.088

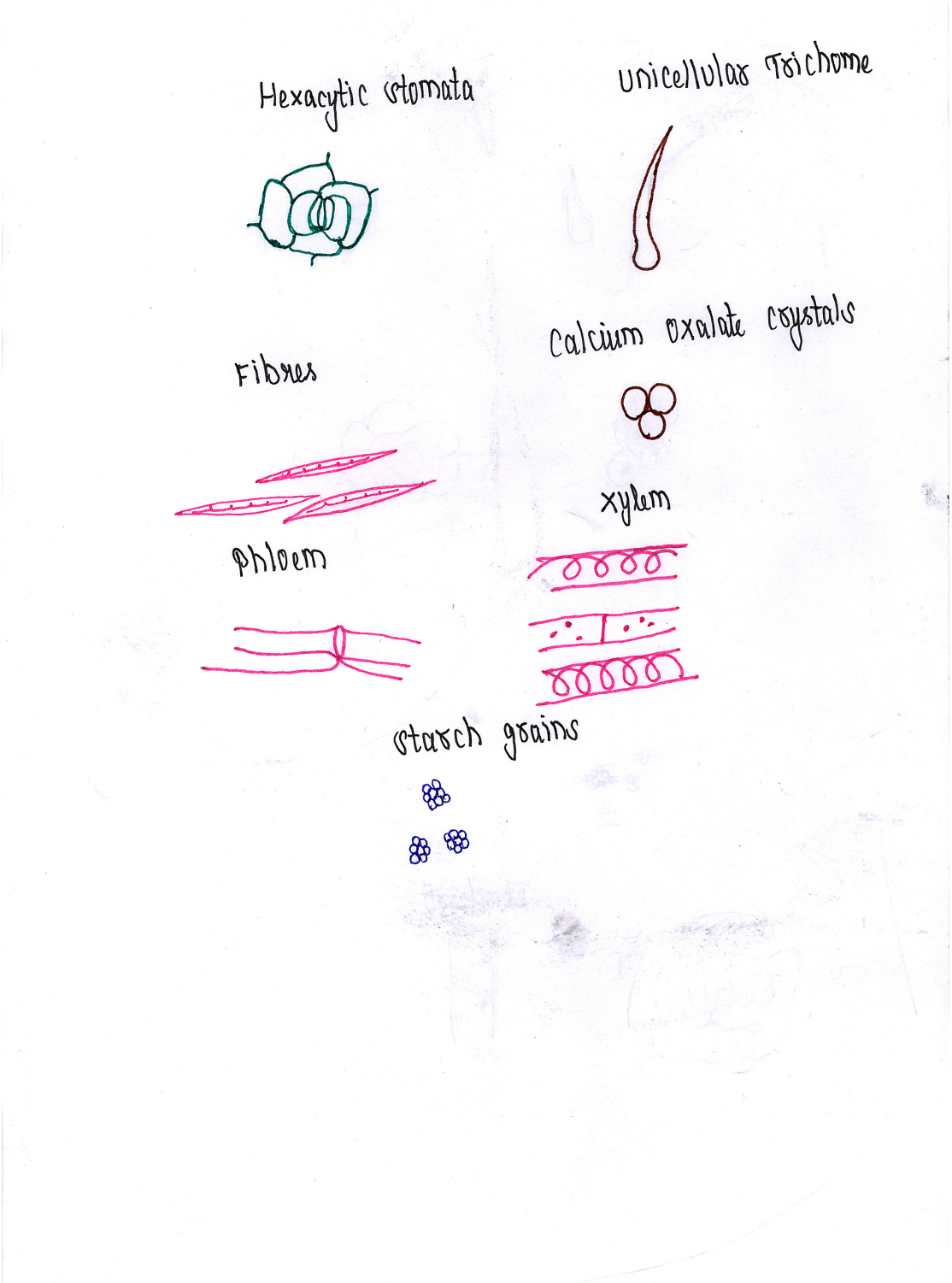
*** mean of 6 readings ± SEM**

SECTION E - POWDER MICROSCOPY AND POWDER ANALYSIS

Powder microscopic characters

The leaf powder of*Costuspictus*D. Don was studied under the microscope and the following inclusions were observed **(Fig. 11)**. The microscopic analysis of the powder of the leaf showed fragments of simple unicellulartrichomes, hexacyticstomata,abaxial solitary bundle has a single, wide, circular thin walled xylem elements and a small clusters of phloem elements located on the upper side.The vascular bundle is surrounded by wide layer of parenchymatous bundle sheath, mesophyll cells containing starch grains, Calcium oxalate

Fig. 11 Powder Microscopy of Costus pictus D. Don



crystals of minute particle are aggregated in to large masses in the leaf mesophyll cells, Tracheids with spiral thickenings and epidermal cells, fibres are observed.

Behaviour of powder with different reagents

The behavior of crude leaf powder of *Costuspictus* D. Don with various reagents is presented in **Table 3.** From the table, it can be observed that the powder with 10% potassium hydroxide under UV light at 254nm exhibited a greenish florescence which will be useful for identification of the plant material in crude form.

Table 3: Behaviour of the *Costuspictus*D. Don powder with various chemical reagents

Drug powder + reagent	Colour in day light (Visible)	Colour in UV light	
		254nm	365nm
Powder	Pale green	Green	Pale green
Powder + 1M sodium hydroxide	Brownish green	Green	Brown
Powder + Iodine	Green	Dark green	Brown
Powder + 10% potassium hydroxide	Light brown	Fluorescent green	Brown
Powder + 1M Hydrochloric acid	Pale green	Dark green	Green
Powder + Glacial acetic acid	Dark green	Dark green	Green
Powder + 50% sulphuric acid	Dark green	Dark green	Green
Powder + 50% nitric acid	Brick red	Green	Green
Powder + 50% hydrochloric acid	Pale green	Dark green	Green

Note: Colour reactions are viewed under natural light by naked eye

Fluorescent analysis of the Extracts

The behaviour of various extracts of Costuspictus D. Don in natural light and under UV light at 254nm and 365nm are presented in **Table 4.** From the table, it was observed that the benzene was orange and ethyl acetate extracts were red under UV light at 254nm. These parameters are useful for quality control and purity checking of the plant in powder form.

Table 4: Fluorescence Analysis of extracts of *Costuspictus*D. Don

Extract	Consistency	Colour in day light	Colour in UV light	
			254nm	365nm
Ethanol	Semisolid	Yellowish green	Brown	Yellowish green
Methanol	Semisolid	Dark green	Brown	Yellow colour
Chloroform	Semisolid	Dark green	Brown	Brown
Ethyl acetate	Semisolid	Dark green	Red	Green
Water	Semisolid	Dark green	Green	Brown
Benzene	Semisolid	Green	Orange	Brownish green
Petroleum ether	Semisolid	Green	Brown	Brown

STANDARDIZATION PARAMETERS

The results obtained for various standardization parameters are presented in **Table 5.**

Table 5: Standardization parameters of *Costuspictus*D. Don

S. No	Parameters*	Values*expressed as %
1	**Volatile oil**	0.96 ± 0.033
2	**Moisture content**	12.38 ± 0.008
3	**Ash values**	
	Total ash	15.28 ± 0.140
	Acid insoluble ash	3.17 ± 0.080
	Water soluble ash	9.70 ± 0.110
4	**Foreign organic matter**	0.06 ± 0.080
5	**Extractive Values**	
	Petroleum ether	25.74 ± 0.150
	Chloroform	7.20 ± 0.300
	Ethyl acetate	8.60 ± 0.030
	Ethanol	5.10 ± 0.110
	Methanol	16.25 ± 0.040
	Water	11.12 ± 0.600
	Benzene	5.76 ± 0.090
6	**Foaming index**	Less than100
7	**Swelling index**	**expressed as mL**
	Initial volume	3.2± 0.10
	Final volume	7.6 ± 0.140

*** mean of three readings ± SEM**

From the **Table 5,** it can be seen that the foreign organic matter present in the crude material was very low. The percentage of total ash was found to be 15.28 ±0.14 and the percentage of water soluble ash was found to be 9.7 ± 0.11, while the acid insoluble ash was found to be 3.17 ± 0.08.

The determination of ash values helps to find out where the powdered material was adulterated with sand and other inorganic material. The water soluble ash helps us to find the amount of inorganic material present in the crude drug while acid insoluble ash helps us to find the amount of sand and other debris in the crude material.

The various extractive values with different solvents have been determined. A maximum extractive value was found with petroleum ether 25.74 ± 0.15 followed by methanol 16.25 ± 0.04. The extractive values help us to decide what solvent will be useful for extraction of maximum active principles and also helps to decide whether the crude material has already been exhausted or not.

The pharmacognostical studies of *Costuspictus* D. Don gave the valuable information regarding the morphology of crude drugs. They can be useful for the authentication of this plant among all species of *Costus*. The microscopic character, leaf constants, quantitative analysis and physico-chemical parameters studied are useful for setting standards for crude drug and to judge the adulteration and purity of this drug. Since the parameters are constant and any change in these values are indicative of substitution and adulteration of the plant materials.

SYNTHESIS OF MECPAgNPs

CHAPTER – 6

SYNTHESIS OF METHANOLIC LEAF EXTRACT OF *Costuspictus*D.Don SILVER NANOPARTICLES [91-101]

Nanoparticles are materials with overall dimensions in the nanoscale. In recent years, these materials have emerged as important players in modern medicine, with applications ranging from contrast agents in medical imaging to carriers for gene delivery into individual cells. Nanoparticles have a number of properties that distinguish them from bulk materials simply by virtue of their size, such as chemical reactivity, energy absorption, and biological mobility.

Nanoparticles can be broadly classified into two groups: Organic nanoparticles and Inorganic nanoparticles. Organic nanoparticle are carbon nanoparticle (fullerenes) and inorganic nanoparticles are magnetic nanoparticles, noble nanoparticles (gold and silver), semiconductor nanoparticles (titanium oxide and zinc oxide).

Metallic nanoparticlesare emerging as new carriers and have been used for a huge number of applications in various areas of medical treatment. Recent advances have opened the way to site-specific targeting and drug delivery by these nanoparticles.

Silver (Ag) a noble metal, has potential applications in medicine due to its unique properties.[91]Silver nanoparticles have attracted research in the field of nanotechnology, due to its distinct properties such as good conductivity, chemically stable, catalytic activity, surface enhanced Raman scattering and antimicrobial activity.[92,93] There are various methods for silver nanoparticles preparation, for example; sol-gel process, chemical precipitation, reverse micelle method, hydrothermal method, microwave, chemical vapor depositionand biological methods, etc.[9] However; biologicalmethods are preferred for being eco-friendly, cost effective, and don't involve the use of toxic chemicals.

The possibility of using plant materials for the synthesis of nanoscale metals was reported initially byGardea-Torresdey. The biosynthesis of nanoparticles, which represents a connection between biotechnology and nanotechnology, has received increasing consideration due to the growing need to developenvironmental friendly technologies for material syntheses. The search for appropriate biomaterials for the biosynthesis of nanoparticles continues through many different synthetic methods.

Plants can be described as nano factories which provide potential pathway to bioaccumulation into food chain and environment. Among the different biological agents plants provide safe and beneficial way to the synthesis of metallic nanoparticle as it is easily available so there is possibilities for large scale production apart from this the synthesis route is eco-friendly, the rate of production is faster in comparison to other biological models such as bacteria, algae and fungi.

In this study, the synthesis and characterization of Ag/*Costuspictus*D. Donby a green method is reported. The Ag-NPs were prepared using silver nitrate as silver precursor and *Costuspictus* leafmethanolic extract as reducing agent and stabilizer.

MATERIALS AND METHODS

All the glasswares were washed with dilute nitric oxide followed by double distilled water and dried in hot air oven.

- **Chemical reagents**

  Methanol

  Silver nitrate ($AgNO_3$)

  Double distilled water

- **Instruments Required**

  Centrifuge (REMI)

  Magnetic stirrer with hot plate

Lyophilizer (Lyodel-Delvac Pumps Pvt. Ltd, USA)

Shimadzu UV-Visible spectrophotometer, Model 1800

Scanning Electron Microscopy (Hitachi X650, Tokyo, Japan)

Procedure

Collection of the leaves

Healthy plant leaves of *Costuspictus*D.Don were collected and cleaned properly in running tap water.

Leaf drying and pulverizing

The leaves were collected and shade dried. It was powdered in a mixer. The powder was sieved in a No.60 sieve and kept in a well closed container in a dry place.

Preparation of methanolic leaf extracts of *Costuspictus* D. Don

About 500g of the dried powdered leaf of *Costuspictus*D. Donwas defatted with 1.5L petroleum ether (60-80^0 C) by maceration. The solvent was removed by filtration and the marc was dried. To the dried marc 1.5L of methanol was added and the extraction was performed by triple maceration (72h process). It was then filtered and the combined filtrate was evaporated to a cohesive mass using rota vapour.

Preparation of stock solution 2mg/20mL

2mg of the methanolic extract was weighed and diluted to 20mL with methanol. It was stored at 4°C until further use.[99] **(Fig.12).**

Preparation of 1mM silver nitrate aqueous solution ($AgNO_3$):

An accurately weighed 0.017g of silver nitrate was dissolved with 100mL of double distilled water and stored in amber colour bottle until further use **(Fig.13).**

Fig. 12: stock solution of MECP 2mg/ 20mL

Fig. 13: (A), (B) & (C)

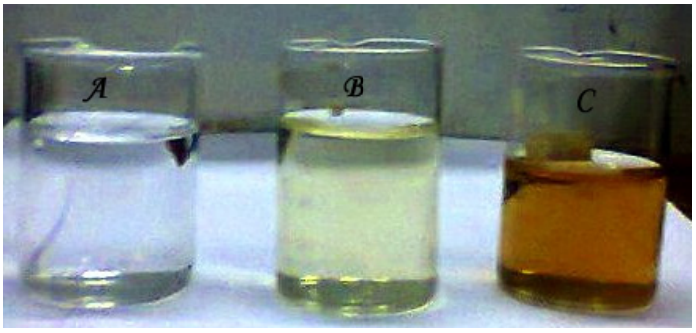


Fig. 13: (A) – Aqueous solution of 1Mm $AgNO_3$

Fig. 13: (B) – Aqueous solution of 1Mm $AgNO_3$ with MECP after zero minutes

Fig. 13: (B) – Aqueous solution of 1Mm $AgNO_3$ with MECP after 5hrs.

Synthesis ofmethanolic leaf extracts of *Costuspictus* D. Don silver nanoparticles (MECPAgNPs)

5mL of the methanolic leaf extract of *Costuspictus*D. Don was taken in the conical flask separately and placed on a magnetic stirrer with hot plate. To this 50mL of 1mM $AgNO_3$ solution was added dropwise with constant stirring 120rpm at 50-60°C. The colour change of the solution was checked periodically. The colour change of the medium from colourless to brown after 5h **(Figs.14.1 to 14.6)**was observed which indicated the formation of silvernanoparticles. It showed that aqueous silver ions could be reduced by the methanolic extract of *Costuspictus*D. Donto generate extremely stable silver nanoparticles.[99]

Seperation of silver nanoparticles

The synthesized silver nanoparticles were separated by centrifuging using a centrifuge at 10,000rpm for 15min. The supernatant liquid was re-suspended in the sterile double distilled water. The process was carried out thrice to get rid of any unco-ordinated biomolecules. After, the desired reaction period, the supernatant liquid was discarded and the pellets were collected and stored at 4°C for further use.[100]

Lyophilization

The pellet obtained was then lyophilized by using freeze dryer (Lyodel-Delvac Pumps Pvt. Ltd, USA) to enhance the stability of silver nanoparticles. The freshly prepared MECPAgNPs are lyophilized with cryoprotective agent (mannitol). Then it was rapidly cooled down to -50^0C for 2h followed by primary drying at 1.03mbar and secondary drying at 0.001mbar.[100] After lyophilization the synthesizedMECPAgNPs was stored at 4°C for further use **(Fig. 15).**

Characterization of synthesized MECPAgNPs

The characterization of synthesized MECPAgNPswas carried out by using the following analytical parameters

Figs. showing the colour change of the medium from colourless to brown colour after 5hrs

Fig. 14.1 At 0 mins 

Fig. 14.2 After 1hr 

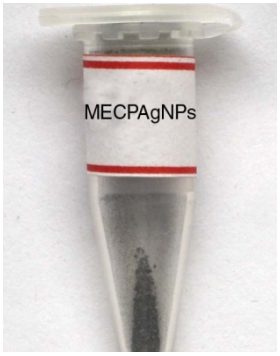
Fig. 14.3 After 2hrs 

Fig. 14.4 After 3hrs 

Fig. 14.5After 4hrs 

Fig. 14.6 After 5hrs 

Fig. 15: Synthesized MECPAgNPs



Particle size

Zeta potential studies

Polydispersity index

UV-Visible spectal analysis

Morphological studies using SEM

Determination of Particle size and Zeta potential

The mean particle size (z-average), polydispersity index (PI) and zeta potential of MECPAgNPswere determined by dynamic light scattering technique using a zeta size analyzer (Nano ZS 90, Malvern Instruments Ltd.,UK). The freeze dried powders were dispersed with water to obtain a proper scattering intensity before measurement.[100] The results obtained are presented in the **Table.6 & Figs.16, 17.**

UV-Visible spectroscopy

The formation and completion of silver nanoparticles was characterized by UV-Visible spectroscopy by using Shimadzu UV- Visible spectrophotometer, Model 1800. The bio-reduction of the Ag^{+} ions in solution was monitored by periodical sampling of aliquots and the UV-Visible spectra of these aliquots were monitored as a function of time of reaction in 200-600nm range operated at a resolution of 1nm. Distilled water was used as a blank.[62] The results obtained are presented in the **Fig. 18.**

Morphological studies of synthesized MECPAgNPsby using Scanning Electron Microscopy (SEM)

Morphological evaluation of theMECPAgNPs was carried out by using scanning electron microscope(SEM) (Hitachi X650, Tokyo, Japan). SEM gave high-resolution images on the surface of the sample. The scanning electron microscope workedon the same principle

of an optical microscope, but it measured the electrons scattered from the sample rather than photon 18.Because electrons can be accelerated by an electric potential, the wavelength can be made shorter than the one of photons. This made the SEM capable of magnifying images up to 200.000 times. At the same time it was possible to achieve high resolution pictures of the surface, making the instrument very useful in determining the morphology and size of nanoparticles. Thin films of a sample prepared on a carbon grid by just dropping a very small amount of the sample on the grid, extra solution was removed by using a blotting paper and then the film on the SEM grid was allowed to dry by keeping it under the mercury lamp for 5 minutes. Further the secondary electron sputtering at an applied potential of20 kV was adopted prior to recording the SEM.[99] The results are depicted in the**Fig. 19.**

RESULTS AND DISCUSSION[102-105]

Synthesis ofMethanolic Leaf extracts of *Costuspictus* D. Don silver nanoparticles (MECPAgNPs)

There was a visible color change after the substrate was added to the plant extract. Initially the plant extract was colourless. Upon adding the silver salt, it turned brown. After 5h, no significant colour change was observed. Increased concentrations of silver nitrate resulted in a brown solution of nanosilver indicating the completion of reaction. Reduction of silver ions into silver nanoparticles using methanolic leaf extract of *Costuspictus*D. Don was evidenced by visual change of colour from colourless to brown colour which indicated the formation of silver nanoparticles due to the excitation of surface Plasmon vibration in silver nanoparticles[102]as shown in **Figs. 14.1 to 14.6.**

Determination of Particle size and Zeta potential

Particle size, size distribution and zeta potential were important characterizations of the silver nanoparticles because they govern the other characterizations, such as saturation solubility and dissolution velocity, physical stability, or even biological performances.[103]

Particle size measurements: Mean particle size diameter and polydispersity indices were all measured in solutions directly after synthesis, using photon correlation spectroscopy (PCS). The size of the colloidal silver nanoparticles, their granulometric distribution has been recorded, expressed against the particles number and their occupied volume.[70]

The average particle size (z-average) is found to be **132.6 nm**. Particle size analysis showed the presence of nanoparticles with polydispersity indices PDI value**0.248** with intercept **0.643.** It is presented in the **Table 6 & Fig. 16.**

Table 6: Mean Particle Size Diameter and Polydispersed Index (PDI) of Bio-synthesized MECPAgNPs

Parameter	Value	Peak No	Peak Size (d.nm)	Peak Intensity %	Peak Width (d.nm)
Z-Average (d.nm)	132.6	Peak 1	141.8	100.0	102.5
PDI	0.248	Peak 2	0.000	0.000	0.000
Intercept	0.643	Peak 3	0.000	0.000	0.000

Fig. 16: Percentageintensity of particle size distribution of bio-synthesized MECPAgNPs

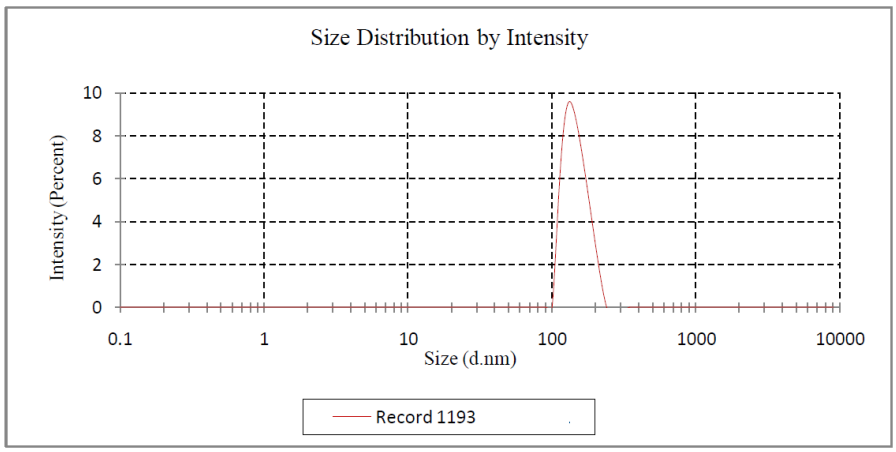
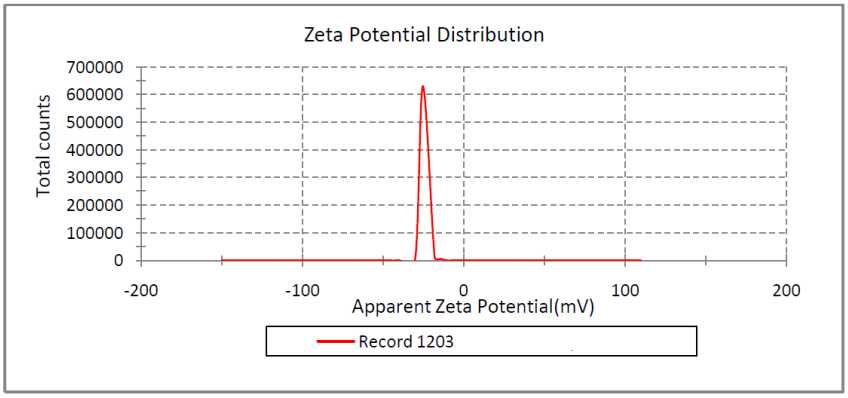


Fig. 17: Zeta potential distribution of bio-synthesized MECPAgNPs



Zeta Potential measurement: a zeta potential was used to determine the surface potential of the silver nanoparticles. Zeta potential is an essential characterization of stability in aqueous silver nanoparticles. A minimum of +30mV zeta potential is required for the indication of stable silver nanoparticles. For the obtained nanoparticles, zeta values were measured and found to be -25.1mV with apeak area of 100% intensity. These values provide full stabilization of the nanoparticles, which may be the main reason in producing particle sizes with anarrow size distribution index.**(Fig. 17)**

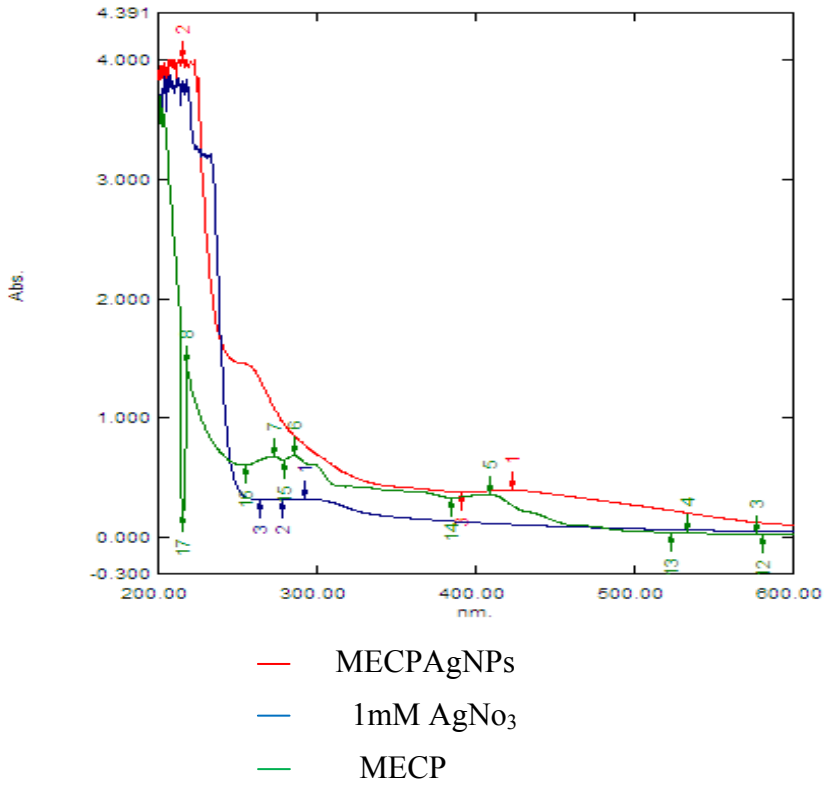
UV-Visible Spectroscopy: The UV-Vis Spectroscopy was the preliminary technique for the characterization of the silver nanoparticles. The reduction of the pure Ag^{+} ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 5 hours (complete colour change) following the dilution of a small aliquot of the sample in distilled water. The UV-Vis spectral analysis was conducted usingShimadzu UV-Vis spectrophotometer, Model 1800 range between 200 and 600 nm.The reduction of silver ions in the aqueous solution of nanoparticles in the solution could be correlated with the respective UV-Vis Spectra of the colloidal solution which exhibited a strong absorption at 420nmas shown in **Fig**. **18.**A typical peak was obtained due to the presence of surface Plasmon resonance silver nanoparticles.[104]

Surface Plasmon resonance: Surface plasmon resonance (SPR) is the collective oscillation of electrons in a solid or liquid stimulated by incident light.

The resonance condition is established when the frequency of light photons matches the natural frequency of surface electrons oscillating against the restoring force of positive nuclei. SPR in nanometer-sized structures is called**localized surface plasmon resonance**.[105]

SPR is the basis of standard tools for measuring adsorption of material onto planar metal (typically gold and silver) surfaces or onto the surface of metal nanoparticles. It is the fundamental principle behind many color-based biosensorapplications and different lab-on-a-chipsensors.

Fig. 18: UV-Vis spectra of MECPAgNPs

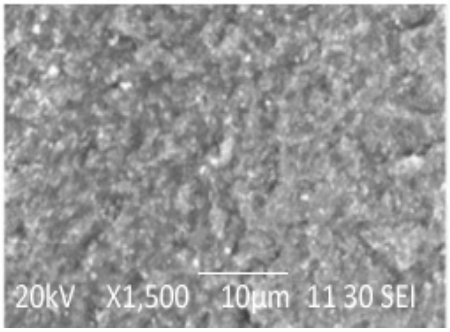


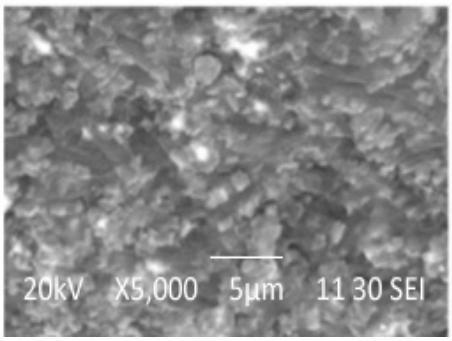
Morphological studies of silver nanoparticles by using Scanning Electron Microscopy (SEM)

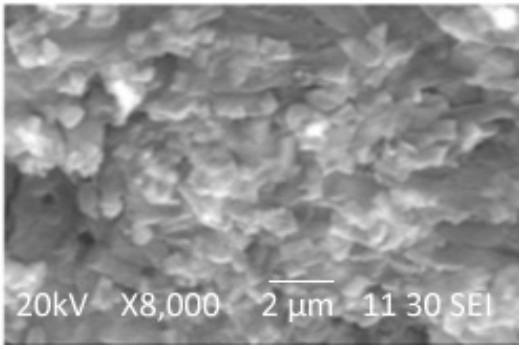
Morphological studies of silver nanoparticles by using Scanning Electron Microscopy (SEM)

A SEM employed to analyze the morphology and size details of the silver nanoparticles that were formed. From (**Fig. 19)** it was showed that the silver nanoparticles formed were spherical in shape, with an average size of around 100nm and uniformly distributed silver nanoparticles on the surface of the cells was observed.

Fig.19: SEM images of MECPAgNPs







PHYTOCHEMICAL EVALUATION

CHAPTER 7

PHYTOCHEMICAL EVALUATION [106-129]

Phytochemistry is the branch of chemistry concerned with plants. It deals mainly with the variety of secondary metabolites that are produced by the plants, their chemical structures, biosynthesis, metabolism, natural distribution and biological functions. Phytochemical evaluation of plant is essential to study the pharmacological activities. It can be done by qualitative chemical analysis using specific reagents for specific constituents followed by confirmation with different chromatographic techniques, like TLC, HPTLC, HPLC, GC etc. Therefore a complete investigation is required to characterize the phytoconstituents qualitatively and quantitatively, before proceeding for its pharmacological and toxicological studies.

MATERIALS AND METHODS

COLLECTION AND PREPARATION OF EXTRACT

The leaves of *Costus pictus* D. Don were collected and washed thoroughly and dried in shade. The shade dried leaves were powdered and sieved in a No.60 sieve and used for the further studies

About 500gof the dried powdered leaf of *Costus pictus* D. Don was defatted with 1.5L petroleum ether (60-80^0 C) by maceration. The solvent was removed by filtration and the marc was dried. To the dried marc 1.5L of methanol was added and the extraction was performed by triple maceration (72h process). It was then filtered and the combined filtrate was evaporated to a cohesive mass using rota vapour.

SECTION A - QUALITATIVE CHEMICAL TESTS FOR THE LEAF POWDER AND CRUDE EXTRACTS [106-110]

Chemical tests can be useful for the investigation of the chemical compounds and to observe the efficiency of an extraction process. The petroleum ether, chloroform, aqueous and methanol extracts were subjected to qualitative chemical tests. The various chemical tests performed on the extracts for steroids, terpenoids, flavones, anthraquinones, sugars, glycosides, alkaloids, quinones, phenols, tannins and saponins and the results were recorded.

1. Test for sterols

The powdered leaf was first extracted with petroleum ether and evaporated to a residue. Then the residue was dissolved in chloroform and tested for sterols.

a. Salkowski's Test

A few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside for while. The red color developing in the lower chloroform layer of the solution indicates the presence of sterols.

b. **Liebermann – Burchard's Test**

To the chloroform solution, a few drops of acetic anhydride and 1mL of concentrated sulphuric acid were added through the sides of the test tube and set aside. At the junction of two layers a brown ring was formed. The upper layer turns green indicates the presence of sterols.

2. Test for terpenoids

A little of the powdered leaf was extracted with chloroform and filtered. The filtrate was warmed gently with tin and thionylchloride. A pink solution indicates the presence of terpenoids.

3. Test for carbohydrates

a. Molisch's Test: To the aqueous extract of the powdered leaf, 2-3 drops of 1% alcoholic solution of α-naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube. A purple colour indicates the presence of carbohydrates.

b. Fehling's Test: To the aqueous extract of the powdered leaf, Fehling's I and II solution was added and heated on a boiling water bath for half an hour. A red precipitate indicates the presence of free reducing sugars.

c. Benedict'sTest: To the aqueous extract of the powdered leaf, an equal volume of Benedict's reagent was added. A red precipitate indicates the presence of reducing sugars.

4. Test for proteins

a. Millon's Test: A small quantity of acidulous – alcoholic extract of the powdered drug was heated with Millon's reagent. A white precipitate turning red on heating indicates the presence of proteins.

b. **Biuret Test:** To one portion of acidulous – alcoholic extract of the powdered drug, 1mL of 10% sodium hydroxide solution, followed by this one drop of dilute copper sulphate solution was added. A violet colour was formed indicating the presence of proteins.

5. Test for flavonoids

a. Shinoda's Test: To the little amount of the powdered drug, few magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for five minutes in a water bath. A red colour indicates the presence of flavonoids.

b. Alkali Test: To the small quantity of test solution, 10% aqueous sodium hydroxide solution was added. A yellow orange colour indicates the presence of flavonoids.

c. Acid Test: A small quantity of test solution was treated with the few drops of concentrated sulphuric acid. A yellow orange colour indicates the presence of flavonoids.

6. Test for alkaloids

About 2g of the powdered material was mixed with 1g of calcium hydroxide and 5mL of water into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water bath. To this 200mL of chloroform was added, mixed well and refluxed for half an hour on a water bath. Then it was filtered and the chloroform was evaporated. To this 5mL of dilute hydrochloric acid was added followed by 2mL of each of the following reagents.

a. Mayer's Reagent: To small quantity of the extract add Mayer's reagent. Cream precipitate indicates the presence of alkaloids.

b. Dragendorff's Reagent: To small amount of the extract add Dragendroff's reagent. Orange brown precipitate indicates the presence of alkaloids.

c. Hager's Reagent: To small quantity of extract add Hager's Reagent. Yellow precipitate indicates the presence of alkaloids.

d. Wagner's Reagent: To small quantity of extract add Wagner's reagent. Reddish brown precipitate indicates the presence of alkaloids.

7. Test for glycosides

Test for Anthraquinone glycosides

Borntrager's Test: The powdered leaf was boiled with dilute sulphuric acid, filtered and, benzene was added to the filtrate and shaken well. The organic layer was separated to which ammonia solution was added slowly. Pink colour in ammoniacal layer shows the presence of anthraquinone glycosides.

Modified Borntrager's Test: About 0.1g of the powdered drug was boiled for 2minutes with dilute hydrochloric acid and few drops of ferric chloride solution were added, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract. Pink colour in ammoniacal layer shows the presence of anthraquinone glycosides.

Test for cardiac glycosides (for deoxysugar)

Keller Kiliani Test: About 1g of the powdered leaf was boiled with 10mL of 70% alcohol for 2min, cooled and filtered. To the filtrate 10mL of water and 5 drops of solution of lead subacetate were added and filtered, evaporated to dryness. The residue was dissolved in 3ml of glacial acetic acid. To this 2 drops of ferric chloride solution was added. Then 3 mL of concentrated sulphuric acid was added to the sides of the test tube carefully and observed. Reddish brown layer indicates the presence of deoxysugars of cardiac glycoside

Test for cyanogenetic glycosides: A small quantity of the powder was placed in a stoppered conical flask with just sufficient water to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2h in a warm place. Brick red color produced on the paper indicates the absence of Cyanogenetic glycosides.

8. Test for saponins

Foam test: About 0.5g of the powdered drug was boiled gently for 2min with 20mL of water and filtered while hot and allowed to cool. 5mL of the filtrate was then diluted with water and shaken vigorously. Frothing indicates the presence of saponins

9. Test for tannins

A small quantity of the powdered drug was extracted with water. To the aqueous extract few drops of ferric chloride solution was added. A bluish black colour indicates the presence of tannins.

10. Test for the presence of Volatile oil

Weighted quantity (250 gm) of fresh leaves were extracted the subjected to hydro distillation using volatile oil estimation apparatus**.** Volatile oil was obtained indicates the presence of volatile oil.

11. Test for Mucilage

A few mL of aqueous extract was prepared from the powdered drug was treated with ruthenium red. A pinkish red colour indicates the presence of mucilage.

12. Test for purine group (Murexide test)

The residue obtained after the evaporation of chloroform as described above was treated with 1mL of hydrochloric acid in a porcelain dish and 0.1gm of potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the vapour of dilute ammonia solution. Purple colour indicates the absence of purine group of alkaloids.

13. Test for Coumarins

a. **$FeCl_3$ test:** To the alcoholic extract of the drug, few drops of alcoholic ferric chloride were added. The formation of deep green color turning yellow on addition of concentrated nitric acid indicates the presence of coumarins.

b. **Flourescence test:** The alcoholic extract of crude extract was mixed with 1N sodium hydroxide solution. A blue-green fluorescence indicates the presence of coumarins.

The above chemical tests were carried out using leaf powder and different plant extracts and the results were tabulated in **Tables 7 & 8** respectively**.** The results obtained for the qualitative chemical tests using leaf powder was presented in **Table 7.**

SECTION B - QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS[111-118]

In quantitative estimations, a particular group of compound present in the crude extracts can be quantified by means of using standard or reference marker compound and then reporting them as equivalent to that much amount of compound present in that extract as per standard compound.

Estimation of total phenolic content

Phenol constitutes the largest group of plant secondary metabolites. Phenols are widespread in nature and are important constituents of medicinal plants. They range from simple structures with one aromatic ring to highly complex polymeric substances such as tannins, flavonoids, anthraquinones and coumarins. Phenolic substances are water soluble and they have been reported to have multiple biological effects, including antioxidant activity.

Principle

The total phenolic content of the methanolic extract of *Costus pictus* D. Don (MECP) and methanolic extract of Costus *pictus* D. Don silver nanoparticles (MECPAgNPs) was determined by Folin-Ciocalteau reagent. This reagent consists of phosphotungstic acids and phosphomolybdic acids mixture. While phenolic content of the extract was oxidized, this reagent is reduced to the mixture of blue molybdenum and tungsten oxides. The intensity of colour is proportional to the amount of phenolic content of the extract and which was measured at 765nm. The total phenolic content in MECP & MECPAgNPs was expressed as milligrams of gallic acid equivalent (GAE) per gm of extract.

Reagents

a) A stock solution of methanolic extract of *Costus pictus* D. Don and methanolic extract of *Costus pictus* D. Don silver nanoparticles (1mg/mL).

b) Folin Ciocalteu Reagent (1N)

Commercially available Folin Ciocalteu Reagent (2N) was diluted with an equal volume of distilled water. The resultant solution was kept in an amber coloured bottle and stored in refrigerator at 4ºC.

c) Sodium carbonate solution (10%w/v)

Procedure

0.5mL and 1mL of the MECP & MECPAgNPs was taken in 10mL volumetric flaskstube. To this, 0.5mL of Folin Ciocalteu reagent (1N) was added and allowed to stand for 15min. Then 1mL of 10% sodium carbonate solution was added. Finally the mixture was mixed with distilled water and made upto10mL, allowed to stand for 30min at room temperature and total phenol was determined by spectrophotometrically at 760nm. The reaction mixture without sample was used as blank. The calibration curve of concentration versus absorbance was generated for gallic acid at different concentrations (2, 4, 6, 8, 10µg/mL) which was used as a standard **(Fig. 20).** The amount of phenol present can be determined by linear regression analysis. The total phenol content of MECP & MECPAgNPs were expressed in terms mg of gallic acid equivalent/g of extract (mg GAE/g) and the results obtained are presented in **Table 9.**

Total flavanoid content estimation

The word "Flavonoid" is derived from the Latin word flavus which means yellow. Most of the flavonoids are indeed yellow in colour. They are widely distributed in nature. It consists of one benzene–gamma – pyrone structure. They have ability to complex with metal

ions and act as an antioxidants and bind to proteins such as structural proteins and enzymes. The different classes with in the groups are distinguished by additional oxygen containing heterocyclic rings and hydroxyl groups which includes flavones, flavanones, flavonols, isoflavones, catechin, anthocyanidins, leuco anthocynadins, chalcones and aurones.

Principle

The aluminium chloride colorimetric technique was used for estimation of total flavonoid content. The aluminium ions form stable complexes with C4 keto group and either to C3 or C5 hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavonoids. The intensity of the colour is proportional to the amount of flavonoids and can be estimated as quercetin equivalent at wavelength of 415nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

a) A stock solution of methanolic extract of *Costus pictus* D. Don and methanolic extract of *Costus pictus* D. Don silver nanoparticles (1mg/mL)
b) 10% aluminum chloride
c) 1M potassium acetate
d) 95%v/v ethanol

Procedure

An aliquot quantity of quercetin was dissolved in ethanol to get a stock solution of 1mg/mL. Further dilutions were made to get concentrations ranging from 20-100µg/mL. 1ml of the above standard solutions were taken in different volumetric flasks, 0.1mL of aluminum chloride solution, 0.1mL of potassium acetate solution and 2.8mL of ethanol were added and

the final volume was then made up to 5mL with distilled water. After 20min the absorbance was measured at 415nm. A sample without aluminium chloride was used as a blank. From the absorbance obtained, a calibration curve was constructed by plotting concentration versus absorbance of quercetin **(Fig. 21).** 0.5mL and 1mL of MECP & MCPEAgNPs of sample at concentrations 100µg/mL and 200µg/mL were taken and the reaction was carried out as above and the absorbance was measured at 415nm after 20min and the readings were tabulated in **Table 10.** The amount of flavonoids present was determined by linear regression analysis. The total flavonoid content in MECP & MCPEAgNPs was expressed as mg of quercetin equivalents per gram of extract.

Estimation of total tannin content

The term "tannin" was first applied by Seguin in 1796. Tannins are complex organic, non–nitrogenous plant products, which generally have astringent properties. These compounds comprise large group of compounds that are widely distributed in the plant kingdom. Most of the tannins are high molecular weight compounds. They are complex polyphenolics, which are polymerisation of simple polyphenols.

Principle

Total tannin content of extract was determined by Folin Denis reagent method. This is based on the stoichiometric oxidation of the molecules containing a phenolic hydroxyl group. Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a highly coloured blue solution. The intensity of which is proportional to the amount of tannins and it was estimated by spectrophotometer at 700 nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

a) A stock solution of methanolic extract of *Costus pictus* D. Don and methanolic extract of *Costus pictus* D. Don silver nanoparticles (1mg/mL)

b) Folin-Denis Reagent: Sodium tungstate 100g and phosphomolybdic acid 20g were dissolved in distilled water 750mL along with phosphoric acid 50mL. The mixture was refluxed for 2h and volume was made up to 1L with distilled water.

c) Sodium carbonate solution (10%)

Procedure

0.1mL and 0.2mL of (1mg/ml) methanolic extracts of MECP & MECPAgNPs was made upto1mL with distilled water in 10mL volumetric flasks. To this, 0.5mL of Folin Denis reagent and 0.8mL of distilled water was added. The flasks were allowed to stand for 15min, then 1mL of sodium carbonate solution (10%) was added to the above mixture and it was made up to 10mL with distilled water. The mixture was allowed to stand for 30min at room temperature and the tannin content was determined spectrophotometrically at 700nm. The reaction mixture without sample was used as blank. Tannic acid at different concentrations (4, 8, 12, 16 & 20µg/mL) was subjected to the above procedure and absorbance noted. A calibration curve was generated by plotting concentration versus absorbance **(Fig. 22).** A linear regression equation was constructed using the calibration curve. The amount of tannin content present can be determined by linear regression analysis. The total tannin content in the MECP & MECPAgNPs was expressed as milligrams of tannic acid equivalent per gm of extract. The results were tabulated in **Table 11.**

Estimation of vitamin C [119-125]

Vitamin C is also known as L-ascorbic acid is a water-soluble vitamin. It is required for the biosynthesis of collagen, L-carnitine, and certain neurotransmitters. It is also involved

in protein metabolism. Collagen is an essential component of connective tissue, which plays a vital role in wound healing. Vitamin C is also an important physiological antioxidant and has been shown to regenerate other antioxidants within the body, including alpha-tocopherol (vitamin E) .Vitamin C might help to prevent or delay the development of certain cancers, cardiovascular disease, and other diseases in which oxidative stress plays a causal role. In addition to its biosynthetic and antioxidant functions, vitamin C plays an important role in immune function and improves the absorption of nonheme iron, the form of iron present in plant-based foods.

Fruits and vegetables are the best sources of vitamin C. Citrus fruits, tomato, amla . Other good food sources include red and green peppers, kiwifruit, broccoli, strawberries, Brussels sprouts, and cantaloupe. Insufficient vitamin C intake causes scurvy, which is characterized by fatigue or lassitude, widespread connective tissue weakness and capillary fragility.

Principle

The estimation of Vitamin C was carried out using the method of *Sarojini et al.,* with slight modifications. The keto group of ascorbic acid undergoes a condensation reaction with 2, 4 dinitro phenyl hydrazine to form a hydrazone which is orange yellow and has an absorbance of about 520nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

a) A stock solution of methanolic extract of *Costus pictus* D. Don and methanolic extract of *Costus pictus* D. Don silver nanoparticles (1mg/mL)

b) 2, 4-dinitro phenyl hydrazine (0.2%)

c) 85% sulphuric acid

Procedure

Ascorbic acid was weighed and dissolved in water to get stock solution of 1mg/mL. Further dilutions were made to get the concentrations ranging from 40-200µg/mL. To 1mL of sample, 0.5mL of 2, 4-dinitro phenyl hydrazine solution was added and incubated for 3h at 37ºC. After 3h, 2.5mL of 85% sulphuric acid was added and the absorbance was measured after 30min at 520nm. A calibration curve was constructed by plotting concentration versus absorbance of ascorbic acid **(Fig. 23).** The procedure was repeated for the MECP & MECPAgNPs as above and the absorbance was measured at 520nm after 3h and the readings were tabulated in **Table 12.** The amount of vitamin C present can be determined by linear regression analysis. The vitamin C content was expressed as mg/g of extract.

SECTION C – CHROMATOGRAPHY [126-129]

Chromatography methods are important analytical tool in the separation, identification and estimation of different components present in crude plant mixture or extract. There are different types of chromatography namely paper chromatography, thin layer chromatography, gas chromatography, high performance thin layer chromatography and high performance liquid chromatography etc.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography is a simple, quick and inexpensive method. It is also called planer chromatography.

Principle

Thin layer chromatography is a technique used for the separation, identification and estimation of single or mixture of components present in the various extracts. It is reliable technique in which solute undergoes distribution between two phases, stationary and mobile

phase. The separation is mainly based on the differential migration that occurs when a solvent flows along the thin layer of stationary phase. This may be achieved by partition and adsorption depending on stationary phase used.[126]

TLC Plate preparation

The plates were prepared using TLC Spreader. 40 gm of silica G was mixed with 85 ml of water to prepare homogenous suspension and poured in the spreader. 0.25mm thickness of plates was prepared and air dried. Then, the dried plates were kept at 110^0C for 30 mins and kept in dessicators.[127]

Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Solvents were analysed in the order of increasing polarity. Factors such as nature of components, stationary phase and mobile phase polarity influence the rate of separation of constituents.

Several mobile phases were tried for the separation of maximum components. From the vast analysis, best solvent was selected which showed good separation with maximum number of components.[128]

Solvent System

Toulene: ethyl acetate: methanol (5:3:2)

Detection

Visual and UV light at 254 & 366nm

The R_f Value of the spots obtained were calculated using the following formula;

$$\textbf{R}_f \textbf{ Value} = \frac{\textbf{Distance travelled by the solute}}{\textbf{Distance travelled by the solvent}}$$

The results obtained for the TLC analysis is presented in the **Table 13 & Fig. 24.**

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

HPTLC is a sophisticated and automated form of TLC. HPTLC is a reliable method for separation of nanogram amount even when present in complex formulation. It is extremely flexible, reliable and cost efficient method in determining the number of ingredients in the mixture.

Basic steps involved in HPTLC are:

Instrument used: CAMAG TLC Scanner 3 "Scanner3-070408"S/N 070408(1.41.21) was used for detection and CAMAG Linomat 5 sample applicator was used for the application of the track. Twin trough plate development chamber was used for development of chromatogram. Software used was winCATS 1.4.3

Sample: The methanolic extract of *Costus pictus* D. Don was dissolved in methanol to get a concentration of 10mg/mL and 2µL of this solution was used for taking HPTLC fingerprint.

Stationary Phase: Aluminium sheets pre-coated with silica gel Merck G F_{254}, 0.2mm layer thickness were used as the stationary phase.

Mobile phase: Toluene: ethyl acetate: methanol (5:3:2) was used as the mobile phase for development of chromatogram. The mobile phase was taken in a CAMAG twin trough glass chamber.

Detection wavelength: The developed plates were examined at wavelength 254nm and 366nm in Densitometry TLC scanner3. The TLC visualization, 3D display of the finger print profile and peak display at 254nm and 366nm are presented in **Figs. 25 to 29.**

The R_f values and area under curve for each peak of are presented in **Table 14.**

RESULTS AND DISCUSSION

SECTION A - QUALITATIVE CHEMICAL TESTS FOR THE LEAF POWDER AND CRUDE EXTRACTS

The results obtained for the preliminary phytochemical screening of the powder is presented in **Table 7.**

Table 7: Preliminary phytochemical screening for the powder of *Costus pictus* D. Don

S. No	Test	Results
1.	**TEST FOR STEROLS**	
	a. Salkowski's test	+
	b. Libermann- burchard's test	+
2.	**TEST FOR TERPENOIDS**	+
3.	**TEST FOR CARBOHYDRATES**	+
	a.Molisch's test	+
	b. Fehling's test	
	c. Benedict's test	+
4.	**TEST FOR PROTEINS**	+
	a. Millon's test	
	b. Biuret test	+
5.	**TEST FOR FLAVONOIDS**	+
	a. Shinoda test	+
	b. Alkali test	+
	c.Acid test	
6.	**TEST FOR ALKALOIDS**	
	a. Mayer's reagent	+
	b. Dragendroff's reagent	+
	c. Hager's reagent	+
	d. Wagner's reagent	+
7.	**TEST FOR GLYCOSIDES**	
	a. Anthraquinone glycosides	-
	i) Borntrager's test	-
	ii) Modified Borntrager's test	-
	b. Cardiac glycosides	+
	i) Keller Killiani test	+
	c. Cyanogenetic glycosides	-
8.	**TEST FOR SAPONINS**	
	Foam test	+
9.	**TEST FOR TANNINS**	
	a.$FeCl_3$ test	+

10.	**TEST FOR VOLATILE OIL**	+
11.	**TEST FOR MUCILAGE**	+
12.	**TEST FOR PURINES**	-
13.	**TEST FOR COUMARINS**	+

(+) indicates positive reaction (-) indicates negative reaction

From the table, it can observed that the powder showed the presence of phytosterols, proteins, carbohydrates, phenolic compounds, alkaloids, flavonoids, cardiac glycosides. It showed absence for cyanogenetic glycosides, anthroquinone glycosides and purines.

The results obtained for the preliminary phytochemical screening of the various extracts are presented in **Table 8.**

Table 8: Preliminary phytochemical screening of various extracts of leaf powder of *Costus pictus* D. Don

S. No	Chemical Tests	Methanol Extract	Chloro form extract	Aqueous Extract
1.	**Test For Sterols**			
	a. Salkowski's test	+	+	-
	b.Libermann-Burchard's test	+	+	-
2.	**Test for carbohydrates**			
	a. Molisch's test	+	-	+
	b. Fehling's test	+	-	+
	c. Benedict's test	+	-	+
3.	**Test for protein**			
	a. Millon's test	+	-	+
	b. Biuret test	+	-	+
4.	**Test for Alkaloids**			
	a.Mayer's reagent	+	+	-
	b. Dragendroff's reagent	+	+	-
	c. Hager's reagent	+	+	-
	d. Wagner's reagent	+	+	-
	e. Test for purine group (Murexide test)	-	-	-
5.	**Test for glycosides**			
	a. Anthraquinone glycosides			
	i) Borntrager's test	-	-	-
	ii) Modified Borntrager's test	-	-	-
	b. Cardiac glycosides			
	i) Keller Killiani test	+	-	-

	ii) Baljet test	+	-	
	iii) Cyanogenetic glycosides	-	-	-
6.	**Test for Saponins**	+	-	+
7.	**Test for Tannins**			
	i) $FeCl_3$ test	+	-	+
	ii) Lead acetate test	+	-	+
8.	**Test for Flavonoids**			
	a. Shinoda test	+	-	-
	b. Alkali test	+	-	-
9.	**Test for Terpenoids**	+	+	-
10.	**Test for Fixed Oils**	-	-	-
11.	**Test for Mucilage**	+	-	+

(+) indicates positive reaction (-) indicates negative reaction

From the **Table 8,** it can observed that the aqueous extract of the *Costus pictus* D. Don showed the presence of flavonoids, tannins, saponins, proteins and carbohydrates while the methanolic extract of *Costus pictus* D. Don showed the presence of flavonoids, phenolic compounds, alkaloids, cardiac glycosides, protein and carbohydrates. The chloroform extract of *Costus pictus* D. Don showed the presence of terpenoids, alkaloids

SECTION B – QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

Estimation of total phenolic content

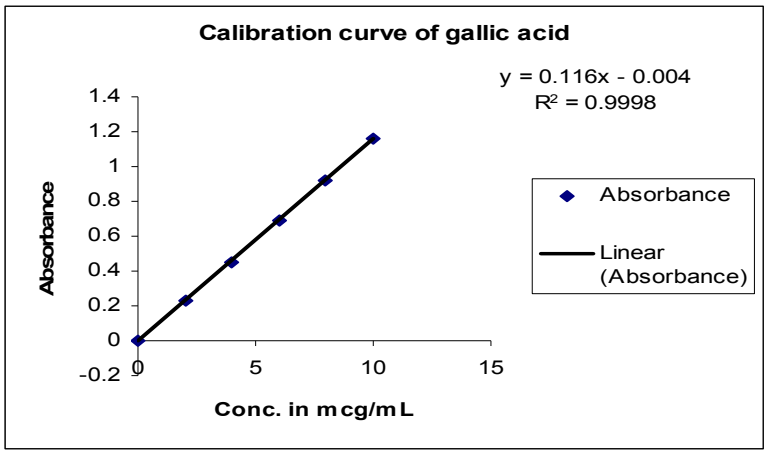
The results for the total phenolic content of MECP & MECPAgNPs are tabulated in **Table 9.**

Table 9: Total phenolic content in MECP & MECPAgNPs in terms of gallic acid equivalents

Conc. of gallic acid in µg/mL	Absorbance at 760nm	Conc. of ethanolic extract in µg/mL	Absorbance at 760nm*		Amount of total phenolic content in terms mgGAE/g of extract*	
			MECP	MEAgNPs	MECP	MEAgNP
2	0.229 ± 0.010	50	0.530±0.001	0.542±0.002	92.06±1.2	94.13±0.3
4	0.452 ± 0.006	100	0.980±0.008	0.991±0.001	84.82±0.5	85.77±0.8
6	0.695 ± 0.005		**Average**		**88.44±0.85**	**90±0.55**
8	0.918 ± 0.031					
10	1.162 ± 0.028					

*** mean of three readings ±SEM**

Fig.20: Calibration curve of gallic acid



The linear regression equation was found to be y = 0.116x-0.004 while the correlation was found to be 0.9998. The amount of phenolic content present in the MECP & MECPAgNPs in terms mg GAE/g of extract was found to be **88.44±0.85** & **90±0.55** by using the above linear regression equation.

Estimation of total flavonoid content

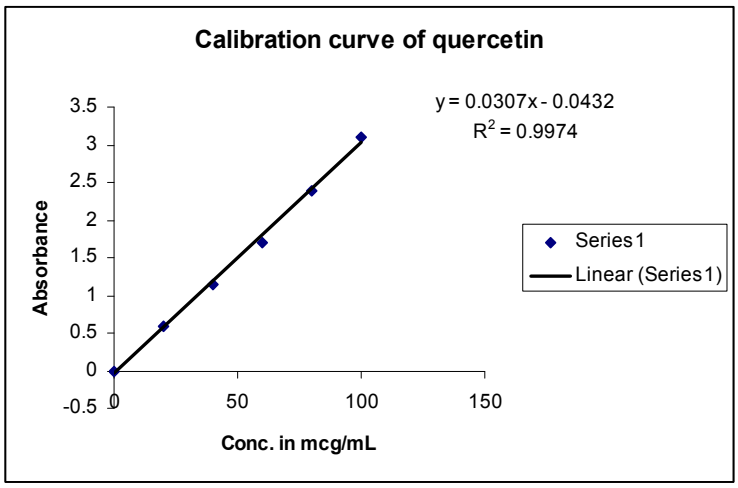
The results for total flavonoid content of MECP & MECPAgNPs are presented in **Table 10.**

Table 10: Total flavonoid content in MECP & MECPAgNPs per gram of in terms of quercetin by aluminium chloride method

Conc. of quercet in in µg/mL	Absorbance at 415nm	Conc. of extract in µg/mL	Absorbance at 415nm*		Amount of total flavonoid content in terms mgQE/g of extract*	
			MECP	MEAgNPs	MECP	MEAgNPs
20	0.589 ± 0.01	100	0.337±0.011	0.35±0.011	115.96±0.26	117.45±0.37
40	1.151 ± 0.04	200	0.669±0.017	0.65±0.017	123.76±3.69	128.07±0.37
60	1.710 ± 0.09		Average		**119.86±1.97**	**122.76±0.37**
80	2.390 ± 0.03					
100	3.112 ± 0.03					

*** mean of three readings ±SEM**

Fig. 21: Calibration curve of quercetin



The linear regression equation was found to be y = 0.0307x-0.0432 while the correlation was found to be 0.9974. The amount of flavonoid content present in the MECP & MECPAgNPs in terms mg quercetin equivalent/g of extract was found to be **119.86±1.97** & **122.76±0.37**mg/g of extract by using the above linear regression equation.

Estimation of total Tannin Content:

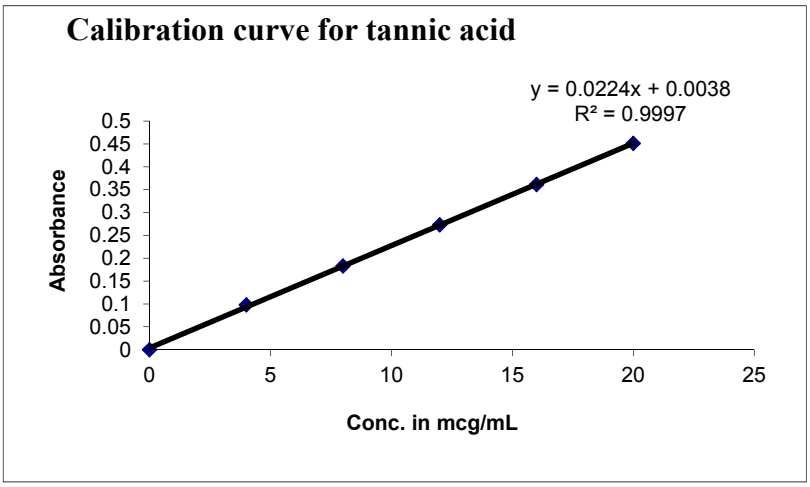
The results for total tannin content of MECP & MECPAgNPs are presented **Table 11.**

Table 11: Total tannin content in MECP & MECPAgNPs in terms of tannic acid equivalents

Conc. of Tannic acid in µg/mL	Absorbance at 700nm	Conc. of extract in µg/mL	Absorbance at 700nm*		Amount of total Tannin content in terms mg tannic acid/g of extract*	
			MECP	MEAgNPs	MECP	MEAgNPs
4	0.098 ± 0.02	10	0.059±0.015	0.07±0.002	246.42±5.15	295.40±9.16
8	0.183 ± 0.01	20	0.140±0.008	0.145±0.005	304.75±1.96	316.66±2.68
12	0.203 ± 0.01		**Average**		**275.58±3.55**	**306.03±13.84**
16	0.361 ± 0.20					
20	0.415 ± 0.10					

*** mean of three readings ±SEM**

Fig-.22: Calibration curve for tannic acid



Total tannin determination is carried out by spectrophotometry after oxidation of the analyte with the Folin–Denis reagent in alkaline medium. This method is based on a redox reaction and other reducing agents in the samples. The linear regression equation was found to be y = 0.022x + 0.003 while the correlation was found to be 0.999. The amount of tannin content present in the MECP & MECPAgNPs in terms of mg tannic acid/g of extract was found to be **275.58±3.55** & **306.03±13.84** by using the above linear regression equation.

Estimatin of Vitamic C Content

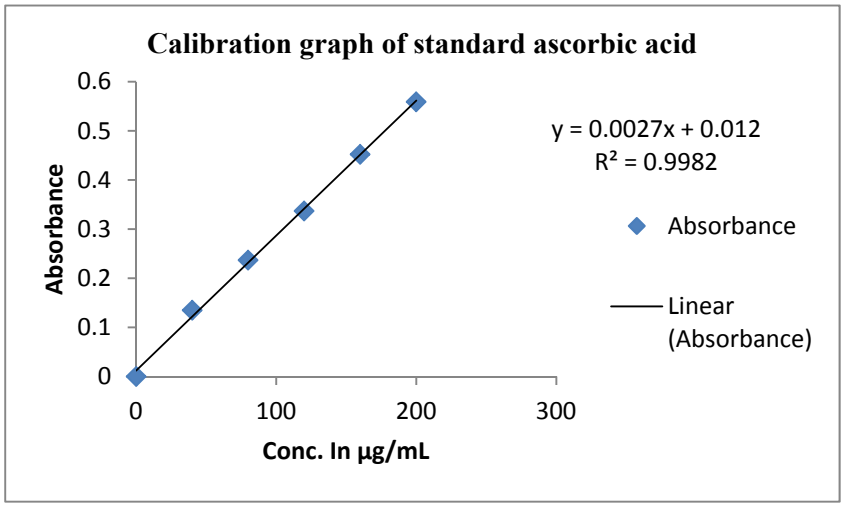
The results for vitamin C content of MECP & MECPAgNPs are presented in **Table 12.**

Table 12: Vitamin C content in MECP & MECPAgNPs

Conc. of ascorbic acid in µg/mL	Absorbance at 520nm	Conc. of ext. in µg/mL	Absorbance at 520nm*		Amount of Vitamin C present/g of extract*	
			MECP	MEAgNPs	MECP	MEAgNPs
40	0.135 ± 0.000	50	0.0866±0.012	0.094±0.066	746.66±12.01	830.04±10.03
80	0.265 ± 0.015					
120	0.346 ± 0.010					
160	0.468 ± 0.011					
200	0.525 ± 0.010					

*** mean of three readings ±SEM**

Fig. 23: Calibration curve of ascorbic acid



The linear regression equation was found to be y = 0.0027x + 0.012 and a correlation Coefficient was 0.9982. The amount of vitamin C content present in the MECP & MECPAgNPs was found to be **746.66±12.01 & 830.04±10.03** mg/gm of extract by using the above linear regression equation.

SECTION C - CHROMATOGRAPHY

THIN LAYER CHROMATOGRAPHY

The results obtained for the TLC analysis with mobile phase is presented in the **Table 13.** The extract showed 5 spots at visible light and 4 spots at 254nm. The R_f value 0.50 when viewed under visible light after development in the mobile phase namely toulene: ethyl acetate: methanol (5:3:2) was dark green which may be due the presence of flavonoid compounds. The R_f values indicating the MECP contains many medicinally active compounds which may be responsible for the therapeutic activity.

Fig: 24: TLC analysis of MECP Visible light
Mobile Phase: Toluene: Ethyl acetate: Methanol (5: 3: 2)
@ Visible light

Table 13: Phytochemical evaluation of Methanolic exract of *Costus pictus* D. Don by TLC studies

S. No	Solvent system	Detecting agent	No of spots	Colour of spots	R_f values
1	Toluene: Ethyl acetate: methanol (5:3:2)	Under visible light	**5**		
			I	Green	0.96
			II	Pale yellow	0.72
			III	Dark yellow	0.68
			IV	Dark green	0.50
			V	Pale green	0.48
		Under 254nm	**4**		
			I	Dark orange	0.74
			II	Orange	0.66
			III	Pale orange	0.52
			IV	Orange	0.46

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

The visualization of the HPTLC plate of MECP at 254nm and 366nm is presented in **Fig.** 25. The photo of plate at 254nm shows the presence of 9 spots while at 366nm shows the presence 10 spots.

Fig. 25: Visualization at 254nm and 366nm

@ 254nm **@366nm**

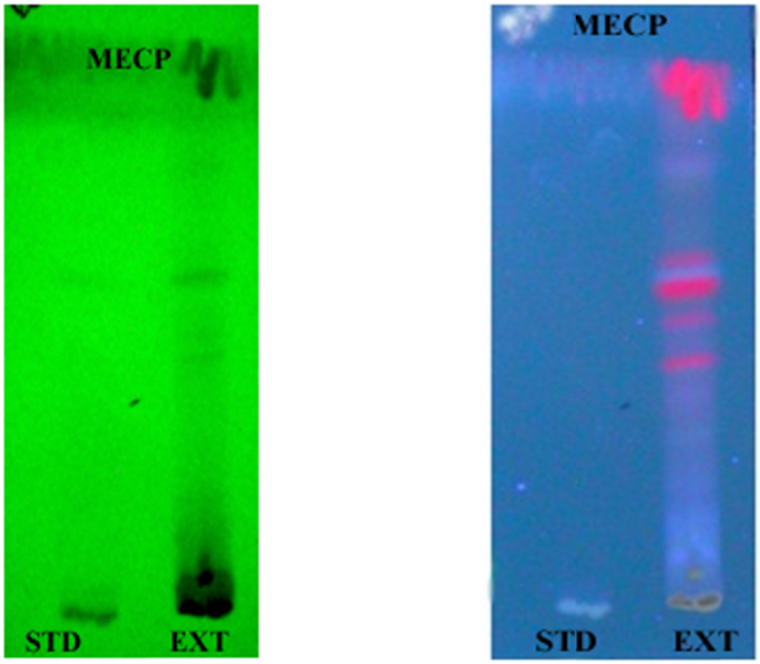


Fig. 26: 3D Display of the fingerprint profile at 254nm and 366nm

@ 254nm **@ 366nm**

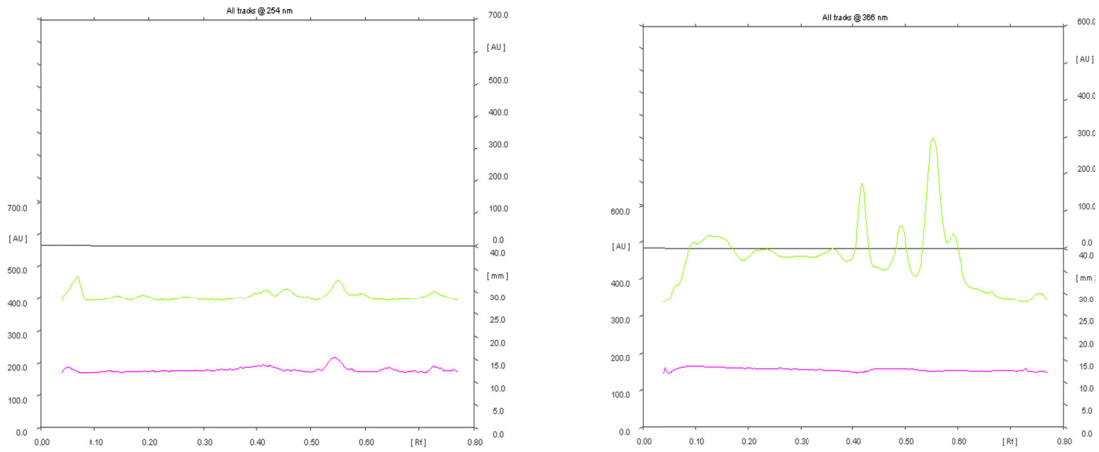
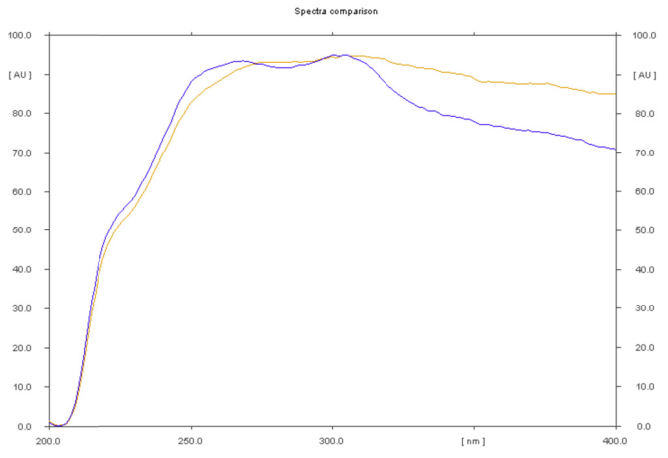


Fig. 27. Overlay spectral analysis

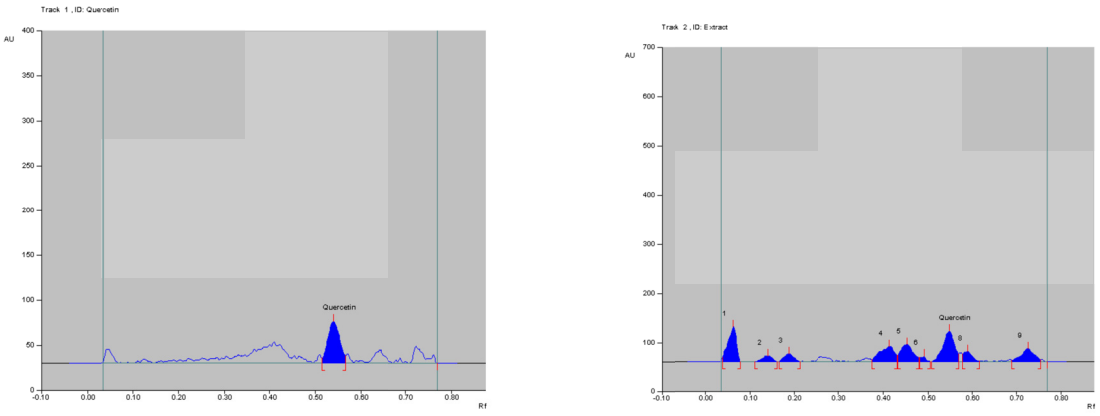


The 3D display of the fingerprint profile and the peak display of MECP at 254nm and 366nm is presented in **Figs. 26 & 27.** The display at 254nm shows the presence of 9 peaks while at 366nm shows the presence 10 peaks. The R_f values of the peaks along with the area under the curve for each peak at 254 and 366 nm are tabulated in **Table 14.**

Fig. 28: Peak display of methanolic leaf extract of Costus pictus D. Don at 254nm and 366nm

@254nm

Quercetin standard **Extract**



@ 366nm

Quercetin standard **Extract**

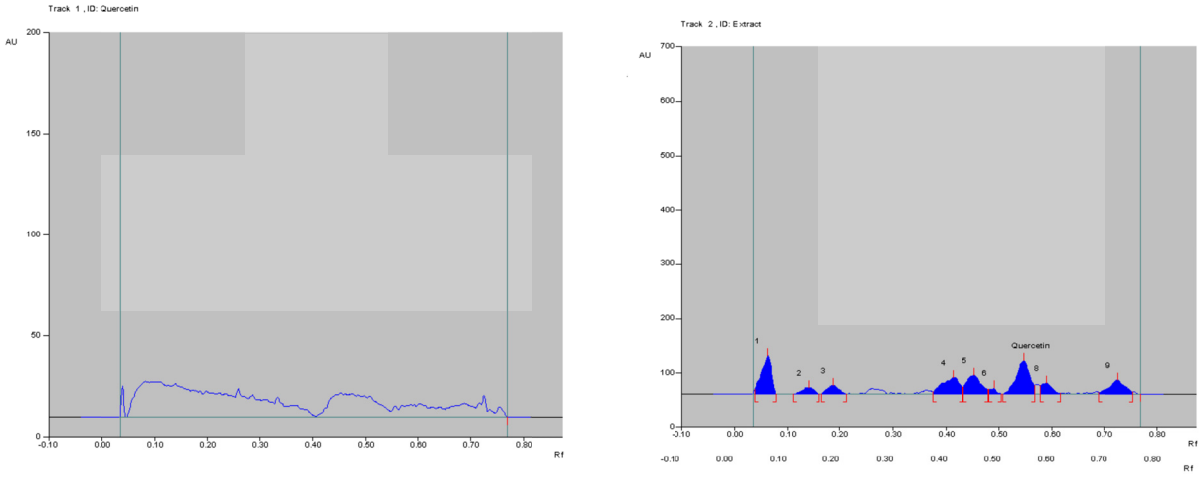


Table 14: R_f values and area under the curve for each peak at 254 & 366nm

S. No	@ 254nm				@ 366nm			
	Querecetin		Sample		Querecetin		Sample	
	Rf value	Area (AU)	Rf value	Area (AU)	Rf value	Area (AU)	Rf value	Area (AU)
1	**0.54**	**1129.7**	0.07	1243.4			0.10	3967.0
2			0.14	249.5			0.13	11131.5
3			0.19	323.7			0.24	8760.9
4			0.42	926.6			0.36	7174.4
5			0.46	894.3			0.42	9075.9
6			0.49	124.6			0.49	6127.3
7			**0.55**	**1485.6**			**0.55**	**12902.2**
8			0.59	383.6			0.59	5217.0
9			0.73	721.9			0.66	610.7
10							0.75	450.5

The HPTLC finger print profile of the MECP Rf values compared with the standard Quercetin. The Rf value of standard quercetin 0.54 while the spot number 7 of the sample has the same Rf value at UV 254nm. Thus the presence of quercetin in MECP is confirmed by this HPTLC method.

PHARMACOLOGICAL EVALUATION

CHAPTER 8

PHARMACOLOGICAL EVALUATION

The word pharmacology is derived from Greek words pharmacon (an active principle) and logos (a discourse or treatise). Pharmacology is the study of drugs and their effects on life processes. It is a fundamental science that sprang to the forefront of modern medicine with demonstrated success in treating diseases and saving lives.

The evaluation of the therapeutic potential of a drug needs to be conducted in a systemic manner. The MECP and MECPAgNPs were subjected to pharmacological screening namely antioxidant and antidibetic activity.

MATERIALS & METHODS

SECTION A – *In- vitro* ANTIOXIDANT ACTIVITY [130-138]

Antioxidants protects the cell against the damaging effects of reactive oxygen species, such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage. They scavenge free radicals generated during oxidative stress and exhibit a protective effect against many diseases like diabetes, cancer, cardiovascular diseases and ageing. The plants contain a wide variety of free radical scavenging molecules such as flavonoids, phenols, terpenoids and vitamins. The natural antioxidants are ascorbic acid, vitamin E, phenolic acids etc. Natural antioxidants tend to be safer, therefore the evaluation of antioxidant activity of various plant extracts is considered as an important step in the identification of their ability to scavenge the free radicals.

Some of the *in vitro* models for the evaluation of antioxidant activity are listed below;

- DPPH assay
- Superoxide scavenging assay

- Hydroxyl radical scavenging assay
- Nitric oxide radical inhibition assay
- Reducing power assay
- Phosphomolybdenum method
- Peroxy nitrile radical scavenging activity
- Xanthine oxidase method
- Ferric reducing ability of plasma
- Thiobarbituric acid assay

Method 1: Free radical Scavenging activity using diphenyl picryl hydrazyl (DPPH)

The free radical scavenging activity of the extracts is evaluated by assessing their ability to reduce the colour of DPPH in ethanol according to Brand Williams. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of specific compound or plant extracts. [131,132]

Principle

A simple method that has been developed to determine the antioxidant activity of plants utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical gives a strong absorption maximum at 517nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 51 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured. [132]

$$DPPH^{\cdot} + AH \rightarrow DPPH - H + A^{\cdot}$$

Instrument

Shimadzu UV Visible spectrometer, Model 1800

Reagents

0.1mM Diphenyl Picryl Hydrazyl Radical in ethanol

Procedure

A stock solution of 0.5mg/mL concentration of MECP & MECPAgNPs was prepared. To the 1mL of various concentrations of test samples, 4mL of DPPH was added. Control was prepared without sample in an identical manner. DPPH was replaced by ethanol in case of blank. The reaction was allowed to be completed in the dark for about 30min. Then the absorbance was measured at 517nm. Vitamin C was used as standard. The percentage scavenging was calculated using the formula **[(Control-Test)/Control] x 100**. A graph was constructed by plotting concentration versus percentage inhibition and a linear regression equation calculated. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A triplicate reading was taken and average was calculated. The results obtained are presented in **Table 15** and **Fig. 30.**

Method 2: Determination of scavenging activity against hydrogen peroxide [136]

Principle

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide probably reacts with Fe^{2+} and possibly Cu^{2+} to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

6% hydrogen peroxide diluted with water in the ratio of 1:10

0.1M phosphate buffer pH 7.4

Procedure

The MECP & MECPAgNPs was dissolved in ethanol to get a stock solution containing 1mg/mL. Varying quantities of the stock solution were added to 3.8mL of 0.1M phosphate buffer solution (pH 7.4) and then 0.2mL of hydrogen peroxide solution was added and the absorbance was measured at 230nm after 10min. The reaction mixture without sample was used as blank. Ascorbic acid was used as standard. The percentage inhibition of hydrogen peroxide was calculated using the formula = $(A_{control} - A_{sample}) / A_{control} \times 100$. The concentration of extract to produce 50% inhibition was found using linear regression analysis. The results obtained are presented in **Table 16** and **Fig. 31.**

Method 3: Total antioxidant activity by Phosphomolybdenum Method [135]

Principle

Total antioxidant capacity was measured by spectrophotometric method of prieto et al. Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) complex at acidic pH and the absorbance was measured at 695nm. This method is used to determine the total antioxidant activity of samples.

$$Mo^{6+} \rightarrow Mo^{5+}$$

Reagents

0.6M sulphuric acid

28mM sodium phosphate

4mM ammonium molybdate

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Procedure

An aliquot of 0.3mL of different concentrations of MECP & MECPAgNPs was combined with 2.7mL of the reagent solution (H_2SO_4, sodium phosphate and ammonium molybdate). In case of blank, 0.3mL of ethanol was used in place of sample. The tubes were incubated for 95°C for 90min. After the mixture was cooled to room temperature, the absorbance was measured at 695nm against blank. The standard Ascorbic acid was treated in a similar manner. The total antioxidant activity is expressed as the number of equivalents of ascorbic acid (µg/g). The results were tabulated in **Table 17** and the graphical representation is presented in **Fig. 32**.

Method 4: Ferric Reducing Antioxidant Power (FRAP) Assay [135]

Total antioxidant activity is measured by FRAP assay of Benzie *et al.,* (1999). The ferric reducing antioxidant power assay measures the potential of antioxidants to reduce the Fe^{3+} and 2,4,6 tripyridyl-s-triazyne (TPTZ) complex present in stoichiometric excess to the blue coloured Fe^{2+} complex which increases the absorption at 593nm.

Principle

At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm. The reaction is non specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

FRAP Reagent

a) Acetate buffer 30mM pH 3.6: Weigh 3.1g sodium acetate trihydrate and add 16 ml of glacial acetic acid and make the volume to 1 L with distilled water.

b) TPTZ (2, 4, 6-tripyridyl-s- triazine) (M.W. 312.34) 10mM in 40mM HCl

c) $FeCl_3$. $6H_2O$ (M.W. 270.30) 20mM

The working FRAP reagent was prepared freshly by mixing a, b & c in the ratio of 10:1:1 at the time of use.

Procedure

50µL, 100µL, 150µL, 200µL, 250µL, 300µL of 1mg/mL concentration of MECP & MECPAgNPs were taken and mixed with 3mL of working FRAP reagent and absorbance was measured at 0min after vortexing at 593nm. Thereafter samples were placed at 37ºC in water bath and absorption was again measured after 4min. Ascorbic acid was used as standard. The FRAP value of the sample was calculated using this equation: [Change in absorbance of sample from 0-4min/change in absorbance of standard from 0-4min] * Frap value of standard. The result obtained for the FRAP assay are presented in the **Table 18** and **Fig. 33.**

Method 5: Reducing power assay [130]

Principle

The reducing power assay is a spectrometric method and is based on the principle that an increase in absorbance of the reaction mixture as concentration increase indicates an

increased antioxidant activity. It is based on the reduction of ferric in potassium ferricyanide to ferrous to form potassium ferrocyanide by the sample and the formation of Prussian blue colour complex when treated with ferric chloride. The absorbance of the blue complex when treated with ferric chloride is measured at 700nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

1% Potassium ferricyanide

10% Trichloro acetic acid.

0.2M, pH 6.6 phosphate buffer

0.1% ferric chloride.

Procedure

About 0.5mL of various concentration of MECP, MECPAgNPs was mixed with 0.75mL phosphate buffer and 0.75mL potassium ferricyanide [K_3 Fe(CN_6)], then the mixture was incubated at 50◦C for 20min. 0.75mL of trichloro acetic acid was added to the mixture, which was then centrifuged at 3000rpm for 10min. Finally 1.5mL of the supernatant solution was mixed with 1.5mL of distilled water and 0.1mL of ferric chloride ($FeCl_3$) and absorbance was measured at 700nm in a UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank. The absorbance of the final reaction mixture of three parallel experiments was expressed as mean ± standard error of mean. Increased absorbance of the reaction mixture indicates stronger reducing power. The results obtained are tabulated in **Table 19** and the graphical representation is presented in **Fig. 34.**

SECTION B – *In- vitro* ANTIDIABETIC ACTIVITY [139-166]

Diabetes is well-known to result in long-term health disorders including cardiovascular disease and blindness. These patients would require the development of several medications with multiple modes of actions. One of the major challenges in the management of diabetes is the monitoring of glucose concentrations. Despite intensive efforts no method is currently available for the continuous non-invasive monitoring of blood glucose. [139-140].

Nanotechnology is a focal point in diabetes research, where nanoparticles in particular are showing great promise in improving the treatment and management of the disease. Due to their ability to potentially enhance drug delivery to areas where there are barriers or unfavourable environments for macromolecules, nanoparticles are being explored as vehicles for improved oral insulin formulations. The use of nanotechnology in the development of glucose sensors is also a prominent focus in non-invasive glucose monitoring systems. There are a few limitations in the use of conventionally available drug delivery systems as pharmacological agents in disease treatment as there is lack of target specificity, altered effects and diminished potency due to drug metabolism in the body. [141,142]

Metallic nanostructures have been studied extensively and are emerging as important colorimetric reporters due to their high extinction coefficients, which are typically several orders of magnitude larger than those of organic dyes. Many researches demonstrated the role of metals in glucose metabolism and the association of their deficiency with diabetes. In particular, nanostructures made from the noble metal silver, with their associated strong plasmon resonance, have generated great interest.

The *in vitro* antidiabetic activity of the methanolic leaf extract of *Costus pictus* silver nanoparticles synthesized was compared with the methanolic leaf extract of *Costus pictus*.was carried out.

The *in vitro* antidiabetic activity was carried out using the following methods;

- ❖ Non – enzymatic glycosylation of haemoglobin assay
- ❖ Inhibition of alpha glucosidase enzyme assay
- ❖ Inhibition of alpha amylase enzyme assay
- ❖ Glucose uptake by yeast cells.

METHOD 1: NON ENZYMATIC GLYCOSYLATION OF HAEMOGLOBIN ASSAY[143-153]

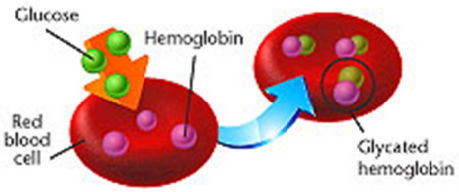
Glycated hemoglobin (Hemoglobin A1c, A1C, or Hb_{1c}; sometimes also **HbA1c**) is a form of haemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time The amount of glycated hemoglobin should not be more than 12%. In Diabetes mellitus, higher amounts of glycated hemoglobin, indicating poorer control of blood glucose levels, have been associated with cardiovascular disease, nephropathy, and retinopathy. Monitoring HbA_{1c} in type 1 diabetic patients may improve outcome of treatment.

Principle

Glycation of proteins is a frequent occurrence, but in the case of hemoglobin, a non enzymatic reaction occurs between glucose and the N-end of the beta chain. This forms a Schiff base which is itself converted to 1-deoxyfructose. This rearrangement is known as Amadori rearrangement. When blood glucose levels are high, glucose molecules attach to the hemoglobin in red blood cells. The longer hyperglycemia occurs in blood, the more glucose binds to hemoglobin in the red blood cells and higher the glycated hemoglobin **Fig. 35.** Glucose levels are intermittently raised in portal vessels carrying absorbed glucose to the liver for regulation. Passing red cells will have increased glycation after sugary food intake. Once a hemoglobin molecule is glycated, it remains that way. A buildup of glycated

hemoglobin within the red cell, therefore, reflects the average level of glucose to which the cell has been exposed during its life-cycle. Measuring glycated hemoglobin assesses the effectiveness of therapy by monitoring long-term serum glucose regulation. The HbA1c level is proportional to average blood glucose concentration over the previous four weeks to three months. Some researchers state that the major proportion of its value is weighted toward the most recent 2 to 4 weeks. This is also supported by the data from actual practice showing that HbA1c level improved significantly already after 20 days since glucose-lowering treatment intensification.

Fig. 35: Glycated haemoglobin



Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

Glucose 2%

Haemoglobin (0.06%)

Gentamycin (0.02%)

0.01M phosphate buffer (pH 7.4)

Preparation of the reagents

Glucose 2%, haemoglobin (0.06%) and gentamycin (0.02%) was prepared in 0.01M phosphate buffer (pH 7.4).

Procedure

Non – enzymatic glycation of haemoglobin assay was assessed using modified method previously described by Bhoomi B. Joshi *et al.,* 2013. The MECP, MECPAgNPs and α-tocopherol was dissolved separately in DMSO to get a stock solution of 1mg/mL. To various concentrations of the stock solution (200-1000µg/mL), 1mL of glucose solution, 1mL of haemoglobin solution and 1mL of gentamycin (20 mg/100 mL) in 0.01M phosphate buffer (pH 7.4) was added. The mixture was then incubated in dark at room temperature for 72 h. α-tocopherol was used as a standard drug. Control was prepared in the similar manner except the addition of extract. The degree of glycosylation of hemoglobin in the presence of different concentration of extracts and their absence were measured at 520nm using UV Visible spectrophotometer.

The percentage inhibition was calculated using the following formula;

$$\% \text{ Inhibition} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

The results obtained are presented in **Table 20** and the graphical representation in **Fig. 36.**

METHOD 2: INHIBITION OF ALPHA GLUCOSIDASE ENZYME ASSSAY[151,152& 158-162]

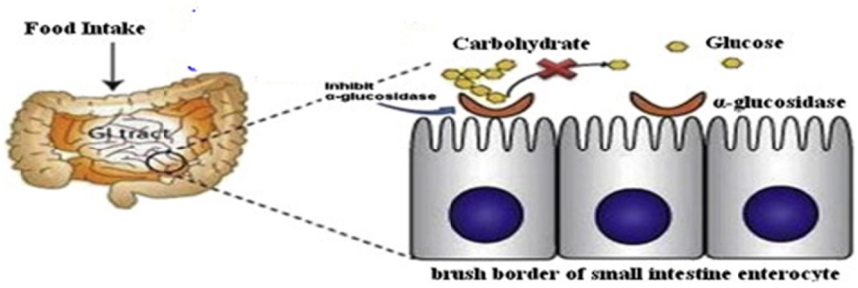
α-glucosidase inhibitors are used to establish greater nhibitors are used to establish greater glycemic control over hyperglycemia in Diabetes type 2 particularly with regard to postprandial hyperglycemia. They may be used as monotherapy in conjunction with an appropriate diabetic diet and exercise, or they may be used in conjunction with other anti-diabetic drugs. α-glucosidase inhibitors may also be useful in patients with Diabetes type 2.

Principle

Alpha glucosidase inhibitors act as competitive inhibitors of enzymes needed to digest carbohydrates specifically α-glucosidase enzymes in the brush border of the small

intestine. The membrane bound intestinal α-glucosidases hydrolyze oligosaccharides, trisaccharides and disacchardies to glucose and other monosaccharides in the small intestine **Fig. 37.** Inhibition of these enzyme systems reduces the rate of digestion of carbohydrates. Less glucose is absorbed because the carbohydrates are not broken down into glucose molecules. In patients with diabetes, the short-term effect of these drugs therapies is to decrease current blood glucose levels: the long term effect is a small reduction in haemoglobin level.

Fig. 37: Inhibition of α-glucosidase enzyme



Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

α-glucosidase enzyme (1U/ml)

0.2M Tris buffer (pH-8)

Glucose oxidase solution

Procedure

α-glucosidase inhibition assay was assessed using modified method previously described by Krishnaveni *et al.,* 1984. To the various concentrations (200-1000µg/mL) of MECP, MECPAgNPs, 0.1mL of alpha-glucosidase enzyme (1U/ml), 1ml of 0.2M Tris buffer

pH 8.0 was added. Then the mixture was incubated for 60min at 35°C. Then the reaction was terminated by heat it for 2min in boiling water bath. The amount of liberated glucose is measured by glucose oxidation method at 540nm using UV Visible spectrophotometer. Acarbose was used as a standard drug. Control was prepared in similar way except extract. Distilled water was used a blank. The percentage inhibition was calculated using the formula

$$\% \text{ Inhibition} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

The experiment was carried out in triplicate and the results obtained are presented in **Table 21** and the graphical representation in **Fig. 38.**

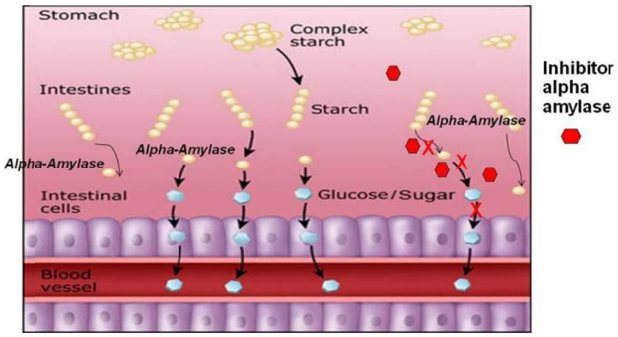
METHOD 3: INHIBITION OF ALPHA AMYLASE ENZYME ASSAY [150-157]

Pancreatic α-amylase belongs to the class of α 1, 4- gluconohydrolases and is one of the important target enzymes for the conventional treatment of diabetes. It catalyzes the initial step in hydrolysis of starch to maltose and maltotriose which are then acted upon by α-glucosidases, broken down in to glucose and it enters the blood stream. Naturally available α-amylase inhibitors from medicinally important plants are shown to be very effective in managing post prandial hyperglycemia which is a major concern in type 2 diabetes.

Principle

Pancreatic α-amylase is a key enzyme in the digestive system and catalyzes the initial step in the hydrolysis of starch, which is a principal source of glucose in the diet. α-amylase inhibitors are agents which inhibit the amylase activity **Fig. 39.** In which results in the delay of carbohydrate digestion and prolong overall carbohydrate digestion time causing reduction in the rate of glucose absorption and consequently reducing the post prandial plasma glucose rise

Fig. 39: Inhibition of α-amylase enzyme



Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

α-amylase enzyme (27.5mg of α-amylase in 100mL of water)

1% w/v of soluble starch

Iodine in potassium iodide solution

0.1M sodium acetate buffer

Preparation of 0.1M sodium acetate buffer

An accurately weighed 820.3mg of sodium acetate and 18.7mg of sodium chloride was dissolved in 100mL of distilled water.

Preparation of Iodine – Iodide indicator

An accurately weighed 635mg 0f iodine and 1gm of potassium iodide was dissolved in 250mL of distilled water.

Procedure

Alpha amylase inhibitory activity was based on the starch iodine method that was originally developed by Abo Baker SM *et al*., 1988. The MECP, MECPAgNPs and acarbose was dissolved in sodium acetate buffer to get a stock solution of 1mg/mL. To various concentrations of the stock solution (200-1000µg/mL), 1mL of 1% w/v of soluble starch solution, 1mL of α-amylase enzyme and 2mL 0.1M sodium phosphate buffer (pH 7.2) was

added. Then this solution was incubated for 1h at 37°C. After incubation, 0.1mL of iodine-iodide indicator was added. The intensity of the colour was measured at 565nm using UV Visible spectrophotometer. 0.1M sodium acetate buffer was used as a blank. The reaction without extract was used as a control. Acarbose was used as a standard drug.Inhibition of enzyme activity was calculated by using the following formula:

$$\% \text{ Inhibition of enzyme activity} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

The results obtained are presented in **Table 22** and the graphical representation is represented in **Fig. 40.**

METHOD 4: GLUCOSE UPTAKE BY YEAST CELLS [162-168]

It is reported that in yeast cells glucose transport is extremely complex and it is generally agreed that glucose is transported in yeast is by a facilitated diffusion process. Facilitated carriers are specific carriers that transport solutes down the concentration gradient. This means that effective transport is only attained if there is removal of intracellular glucose.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

Baker's yeast

Glucose (5 & 10mM)

Glucose oxidase solution

Preparation of yeast suspension

Yeast suspension was prepared according to the method of Cirillo, 1962. 1g of the commercial baker's yeast was washed by repeated centrifugation at 3,000 rpm for 5mi in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water.

Procedure

Glucose uptake by yeast cells assay was assessed using Manjunatha *et al.,* 2008 with slight modification. Various concentrations (40–200µg/mL) of the MECP and MECPAgNPs were added to 1mL of glucose solution (5 & 10mM) and incubated together for 10min at 37°C. The reaction was started by adding 100µL of yeast suspension, vortexed and further incubated at 37°C for 60min. After 60min, the tubes were centrifuged at 2,500rpm for 5min. To 100µL of glucose oxidase solution, 500µL of supernatant solution was added and kept for half an hour at room temperature. Then the percentage increase in glucose uptake by the yeast cells was measured at 520nm using a UV visible spectrophotometer. Distilled water was used as a blank. Acarbose was taken as standard drug. The reaction without extract was used as control. The percentage increase in glucose uptake by yeast cells was calculated using the following formula,

$$\textbf{Increase in glucose uptake (\%)} = \frac{\textbf{Abs sample} - \textbf{Abs control}}{\textbf{Abs sample}} \textbf{X 100}$$

The results obtained are presented in **Table 23** and the graphical representation in **Fig. 41 & 42.**

RESULTS AND DISCUSSION

SECTION A - *In-vitro* ANTIOXIDANT ACTIVITY

The *in-vitro* antioxidant activity of the methanolic extract of *Costus pictus* leaves was evaluated by five methods. The results obtained for these methods are presented in **Tables 15 to 19** and the graphical representations are presented in **Figs. 30 to 34.**

Method I: Free radical Scavenging activity using 2, 2-Diphenyl-1-Picryl hydrazyl (DPPH)

The results obtained for the free radical scavenging activity against DPPH radical is presented in **Table 15** and the graphical representation is presented in **Fig. 31**

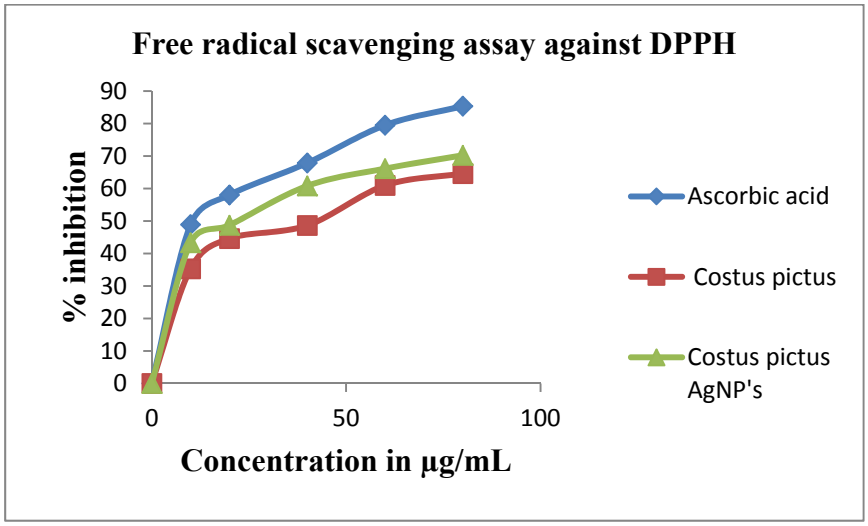
Table 15: Percentage inhibition of MECP, MECPAgNPs & standard ascorbic acid against DPPH at 517nm

S. No	Conc. in µg/mL	Percentage inhibition by ascorbic acid	Percentage inhibition by *Costus pictus*	percentage inhibition by *Costus pictus* AgNP's
1	10	48.91 ± 0.60	35.31±0.115	43.3±0.085
2	20	48.91 ± 0.60	44.62±0.115	48.71±0.066
3	40	67.86 ± 0.27	48.64±0.394	60.82±0.085
4	60	79.49 ± 0.30	60.95±0.167	66.14±0.085
5	80	85.36 ± 0.29	64.61±0.227	70.23±0.182
	IC_{50}	**27.29 µg/ml**	**46.66µg/mL**	**37.64µg/Ml**

***mean of three readings ± SEM**

From the table, it can seen that the MECP, MECPAgNPs showed a percentage inhibition of 64.61±0.227 & 70.23±0.182 while ascorbic acid showed a percentage inhibition of 85.31±0.29 at a concentration of 80µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be **46.66, 37.64** and **27.29µg/mL** for MECP, MECPAgNPs and ascorbic acid respectively **(Fig.30).** From this, MECPAgNPs showed higher antioxidant activity than MECP. The extract possessed a good radical scavenging capacity

Fig.30: Free radical scavenging of MECP, MECPAgNP's and ascorbic acid against DPPH at 517nm



Method 2: Determination of scavenging activity against hydrogen peroxide

The results obtained for the scavenging activity against hydrogen peroxide are presented in **Table 16** and the graphical representation is presented in **Fig. 31**

Table16: Percentage inhibition of hydrogen peroxide by MECP, MECPAgNPs & standard ascorbic acid

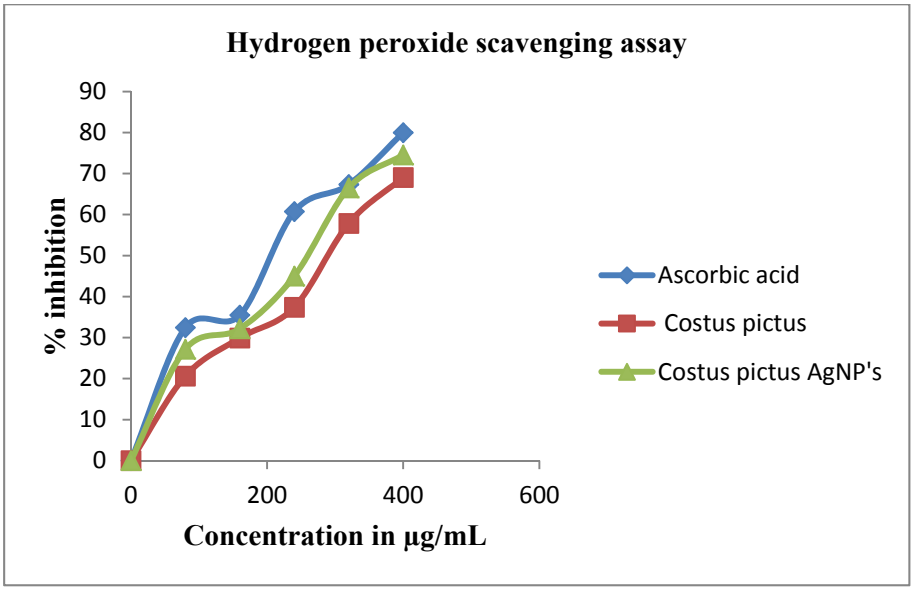
S. No	Conc. in µg/Ml	Percentage inhibition by ascorbic acid	Percentage inhibition by *Costus pictus*	percentage inhibition by *Costus pictus* AgNP's
1	80	32.44 ± 0.81	23.61±0.35	27.16±0.28
2	160	35.47 ± 0.91	32.9±0.46	32.17±0.83
3	240	60.73 ± 0.51	43.45±0.28	45.01±0.43
4	320	67.32 ± 0.53	64.36±0.33	66.49±0.21
5	400	79.99 ± 0.51	74.07±0.29	74.56±0.28
	IC_{50}	**221.21 µg/mL**	**287.14 µg/mL**	**251.82 µg/mL**

***mean of three readings ± SEM**

From the table, it can seen that the MECP, MECPAgNPs showed a percentage inhibition of 74.07 ± 0.29 & 74.56 ± 0.28 while ascorbic acid showed a percentage inhibition of 79.99 ± 0.51 at a concentration of 400µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be**,** **287.14**, **251.82** and **221.21µg/mL** for MECP,

MECPAgNPs and ascorbic acid respectively. From this, MECPAgNPs showed higher antioxidant activity than MECP.

Fig. 31: Scavenging activity by hydrogen peroxide method



Method 3: Antioxidant activity by Phosphomolybdenum method

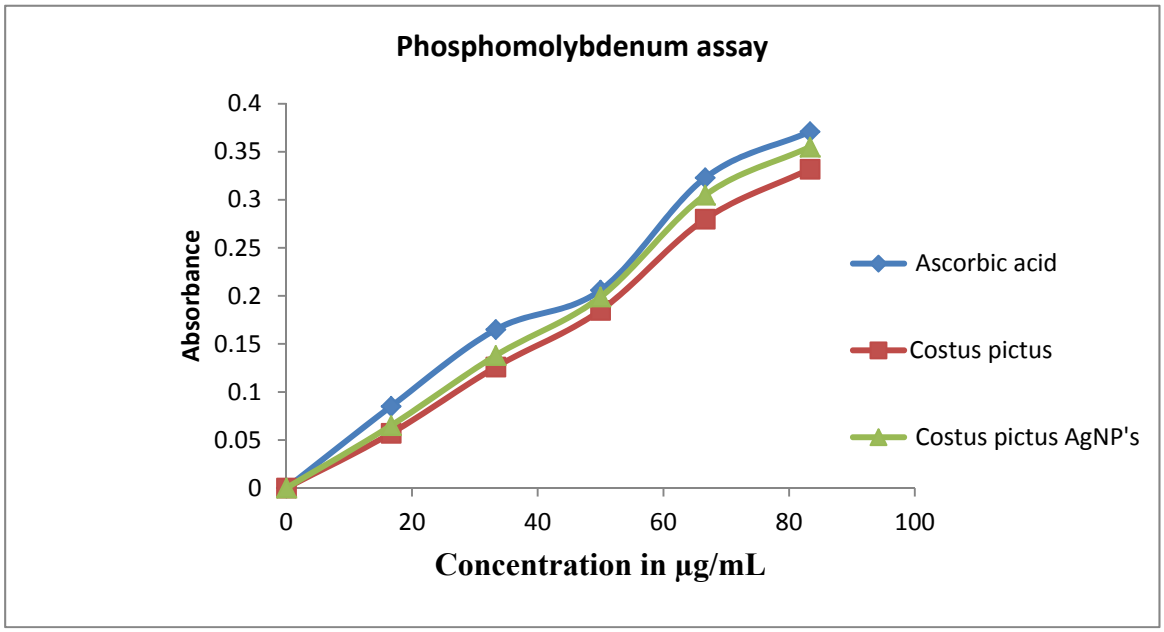
The results obtained for the Phosphomolybdenum activity of MECP, MECPAgNPs and standard ascorbic acid are tabulated in **Table 17** and the graphical representation is presented in **Fig. 32**

Table 17: Absorbance of MECP, MECPAgNPs and standard ascorbic acid by Phosphomolybdenum method

S. No.	Conc. in µg/mL	Absorbance of Ascorbic acid	Absorbance of *Costus pictus*	Absorbance of *Costus pictus* AgNP's
1	16.66	0.085 ± 0.005	0.057±0.003	0.065±0.008
2	33.33	0.165 ± 0.004	0.126±0.005	0.138±0.003
3	50.00	0.206 ± 0.008	0.185±0.006	0.199±0.005
4	66.66	0.323 ± 0.004	0.28±0.008	0.305±0.005
5	83.33	0.371 ± 0.005	0.332±0.005	0.355±0.007

***mean of three readings ± SEM**

Fig. 32: Antioxidant activity by Phosphomolybdenum method



The results obtained for the phosphomplydenum assay are presented in **Table 17.** From the table, it can be seen that the MECP, MECPAgNPs showed an absorbance of 0.332±0.005 & 0.355±0.007 at a concentration of 100µg/mL while ascorbic acid showed an absorbance of 0.371 ± 0.005 at a concentration of 100µg/mL. The extract shows a dose dependent reducing ability. The MECPAgNPs showed higher antioxidant activity than MECP. The graphical representations of the phosphomolybdenum assay of the MECP, MECPAgNPs and ascorbic acid are presented in **Fig. 32.**

Method 4: Ferric Reducing Antioxidant Power (FRAP) Assay

The results obtained for the Ferric Reducing Antioxidant Power assay are presented in **Table 18.** The graphical representations of the reducing power activity of the MECP, MECPAgNPs and ascorbic acid are presented in **Fig. 33.**

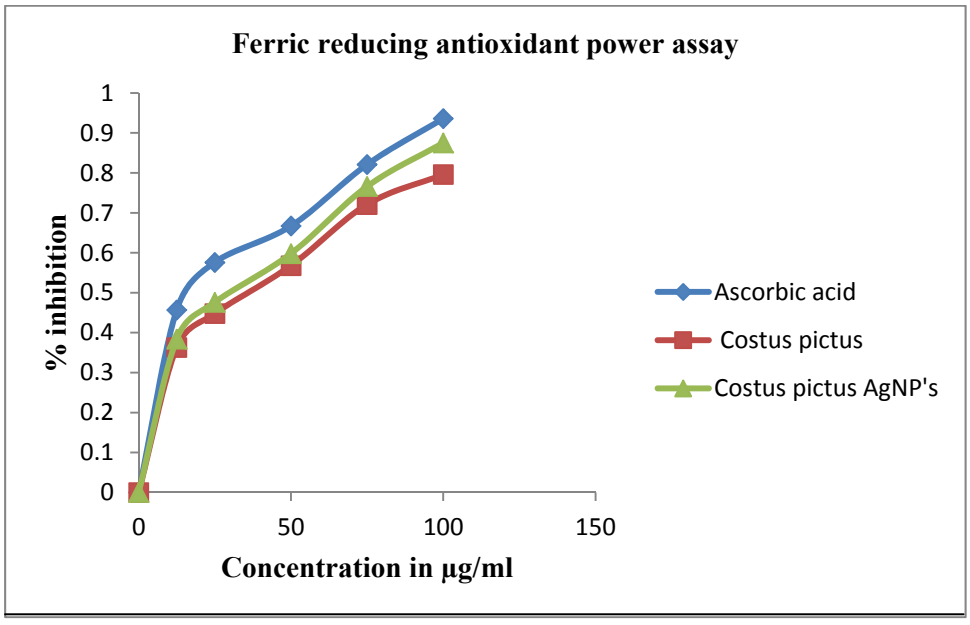
Table 18: Ferric reducing anti-oxidant power assay of MECP, MECPAgNPs & standard ascorbic acid

S. No.	Conc. in µg/mL	Absorbance of Ascorbic acid	Absorbance of *Costus pictus*	Absorbance of *Costus pictus* AgNP's
1	12.5	0.457 ± 0.001	0.36±0.0006	0.38±0.0014
2	25	0.576 ± 0.004	0.44±0.0017	0.47±0.0060
3	50	0.667 ± 0.003	0.57±0.0017	0.59±0.0043
4	75	0.821 ± 0.001	0.72±0.0014	0.76±0.0072
5	100	0.936 ± 0.002	0.79±0.0011	0.87±0.0020

***mean of three readings ± SEM**

From the table, it can be seen that the MECP, MECPAgNPs showed an absorbance of 0.7 ± 0.0011 and 0.87 ± 0.002 at a concentration of 100µg/mL while ascorbic acid showed an absorbance of 0.936 ± 0.002 at a concentration of 100µg/mL. The extract and the nanoparticles showed a dose dependent reducing ability. The nanoparticles at the same concentration showed much better activity than the extract alone.

Fig.33: Ferric reducing anti-oxidant assay of MECP &MECPAgNPs



Method 5: Reducing Power Assay:

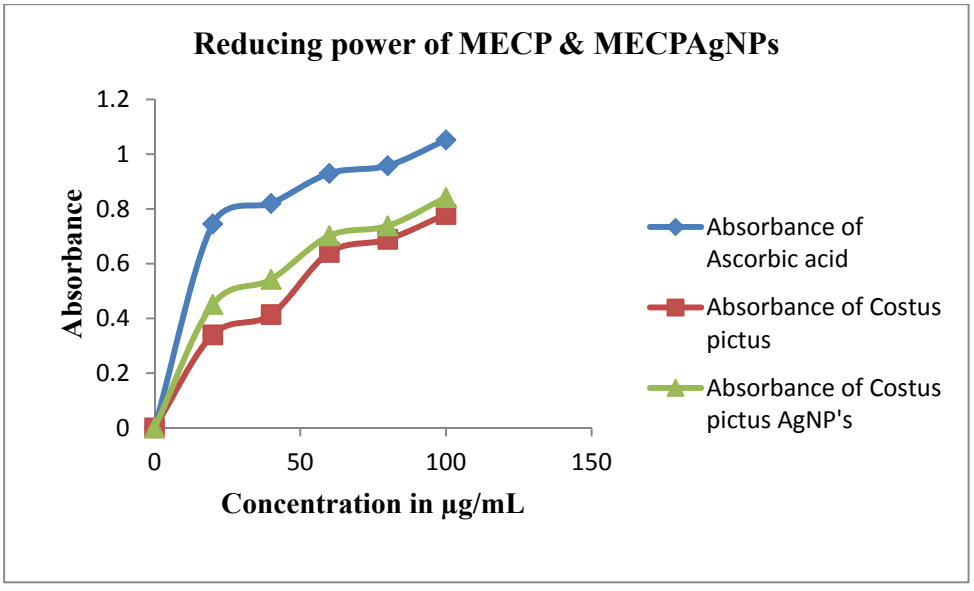
The results obtained for the Reducing Power assay of MECP, MECPAgNPs and standard ascorbic acid are presented in **Table 19** and the graphical representation is presented in **Fig. 34.**

Table 19: Reducing Power Assay of MECP, MECPAgNPs and standard ascorbic acid

S. No.	Conc. in µg/mL	Reducing power of Ascorbic acid	Reducing power of *Costus pictus*	Reducing power of *Costus pictus* AgNP's
1	20	0.745 ± 0.012	0.340±0.002	0.451±0.005
2	40	0.820 ± 0.003	0.414±0.004	0.543±0.004
3	60	0.930 ± 0.002	0.641±0.002	0.702±0.003
4	80	0.958 ± 0.059	0.689±0.003	0.738±0.005
5	100	1.052 ± 0.007	0.780±0.007	0.842±0.007

***mean of three readings**

Fig. 34: Antioxidant activity by reducing power assay



From the **Table 19**, it can be seen that the MECP, MECPAgNPs and ascorbic acid showed the absorbance of **0.780±0.007, 0.842±0.007 & 1.052±0.007** at a concentration of 100µg/mL. The MECPAgNPs showed higher antioxidant activity than MECP.

SECTION B – *In- vitro* ANTIDIABETIC ACTIVITY

METHOD 1: NON ENZYMATIC GLYCOSYLATION OF HAEMOGLOBIN ASSAY

The results obtained for the non-enzymatic glycosylation of haemolglobin assay are presented in the **Table 20** and the graphical representation in **Fig. 36.**

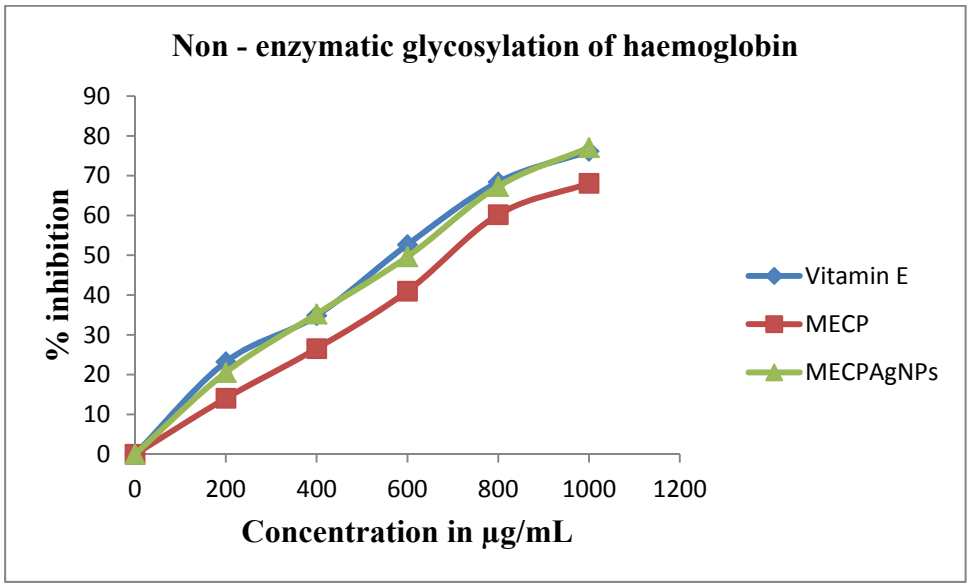
Table 20: Non –enzymatic glycosylation of haemolglobin assay

Conc. µg/ mL	α-tocopherol		MECP		MEAgNPs	
	Absorbance at 520nm*	% Inhibition	Absorbance at 520nm*	% Inhibition	Absorbance at 520nm*	% Inhibition
200	0.159±0.003	23.19±1.674	0.142±0.001	14.06±0.698	0.148±0.001	20.55±0.643
400	0.187±0.003	34.84±1.016	0.163±0.003	26.56±0.313	0.191±0.008	35.34±0.328
600	0.258±0.005	52.68±0.813	0.193±0.008	40.99±0.288	0.22±0.0014	49.62±0.560
800	0.386±0.004	68.38±0.343	0.323±0.009	60.20±1.015	0.373±0.001	67.31±0.155
1000	0.512±0.005	76.16±0.215	0.394±0.002	68.08±0.137	0.520±0.006	77.08±0.100
IC_{50}	**600.26 µg/mL**		**710.71 µg/mL**		**609.61 µg/mL**	

*** mean of three readings ±SEM**

From the **Table 20,** it can be observed that the MECPAgNPs possess very good antidiabetic activity as compared to the MECP .The percentage inhibition of glycosylation is dose dependent, as dose increases, inhibition increases as shown in the **Fig**. **36**. The percentage of inhibition at the concentrations of 200, 400, 600, 800 & 1000 µg/ml by the MECPAgNPs and MECP showed a concentration-dependent reduction. The highest concentration 1000 µg/ml of MECPAgNPs, MECP and α-tocopherol showed a maximum inhibition of 77.08 ± 0.100, 68.0 8± 0.137 & 76.16 ± 0.215 while the lowest concentration 200µg/mL of MECPAgNPs, MECP and α-tocopherol showed a minimum inhibition of 23.19 ± 1.674, 14.06 ± 0.698 & 20.55 ± 0.693 respectively. The IC_{50} values of the MECP, MECPAgNPs and α-tocopherol were found to be 710.71, 609.01 & 600.26µg/mL respectively.

Fig. 36: Non - enzymatic glycosylation of haemoglobin assay



The plant extracts play an important role in the inhibition of the glycosylation end products. An increase in the glycosylation was observed on incubation of hemoglobin with the increasing concentration of the glucose over a period of 72h. However, the plant extracts significantly inhibited the haemoglobin glycosylation which is indicated by the presence of increasing concentration of haemoglobin. MECPAgNPs exhibited higher inhibition of glycosylation as compared with the MECP. The plant extracts also displayed the inhibition of haemoglobin glycosylation at different physiological concentrations of the glucose over the period of 72h, indicating that the plant extracts decreases the formation of the glucose-haemoglobin complex and thus amount of free haemoglobin increases. The activity of MECPAgNPs was found to be very effective than MECP which was depicted in the **Table 20 & Fig. 36**

METHOD 2: INHIBITION OF ALPHA GLUCOSIDASE ENZYME ASSSAY

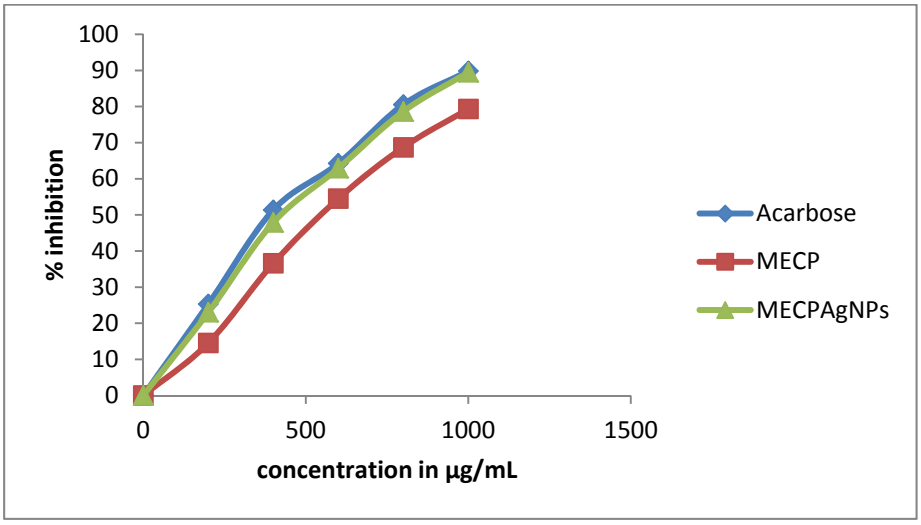
The results obtained for the Inhibition of alpha glucosidase enzyme assay are presented in the **Table 21** and the graphical representation in **Fig. 38.**

Table 21: Inhibition of alpha glucosidase enzyme assay

Conc. µg/ mL	Acarbose		MECP		MEAgNPs	
	Absorbance at 540nm*	% Inhibition	Absorbance at 540nm*	% Inhibition	Absorbance at 540nm*	% Inhibition
200	0.801±0.001	25.30±0.20	0.952±0.006	14.54±0.64	0.842±0.004	23.03±0.45
400	0.521±0.002	51.35±0.19	0.781±0.005	36.60±0.05	0.679±0.001	47.89±0.50
600	0.381±0.002	64.25±0.22	0.506±0.002	54.51±0.19	0.45±0.0017	62.91±0.17
800	0.208±0.004	80.53±0.39	0.399±0.008	68.71±0.08	0.345±0.001	78.64±0.29
1000	0.105±0.002	89.81±0.09	0.204±0.001	79.32±1.95	0.177±0.001	89.47±0.14
IC_{50}	482.68 µg/mL		596.78 µg/mL		501.34 µg/mL	

*** mean of three readings ±SEM**

Fig. 38: Inhibition of alpha glucosidase enzyme assay



From the **Table 21,** it can be observed that the MECPAgNPs possess very good antidiabetic activity as compared to the MECP. The *in vitro* α-glucosidase inhibition study showed that both MECPAgNPs and MECP inhibited α-glucosidase. The percentage inhibition at the α-glucosidase concentrations of 200, 400, 600, 800 & 1000 µg/ml by the

MECPAgNPs and MECP showed a concentration-dependent reduction. The highest concentration 1000µg/mL of MECPAgNPs, MECP and acarbose showed a maximum inhibition of 89.47±0.142, 79.32±1.954% and 89.81±0.093 while the lowest concentration 200µg/mL of MECPAgNPs, MECP and Acarbose showed a minimum inhibition of 25.3±0.197, 14.54±0.64 & 25.3±0.117. **Fig.38.** MECPAgNPs showed strong α-glucosidase inhibition as compared with MECP. The MECP was less potent in inhibiting α-glucosidase as compared to acarbose and MECPAgNPs. The IC_{50} values of the MECP, MECPAgNPs and Acarbose were found to be 596.78 µg/mL, 501.34 µg/mL & 482.68 µg/mL respectively.

α-glucosidase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide and starch to yield glucose. α-glucosidase inhibitors bind to alpha-bond of polysaccharide and prevent the breakdown of polysaccharide to glucose. In our experimental study it was observed that that MECPAgNPs demonstrated significant α-glucosidase inhibition activity as compared to standard drug acarbose.

METHOD 3: INHIBITION OF ALPHA AMYLASE ENZYME ASSSAY

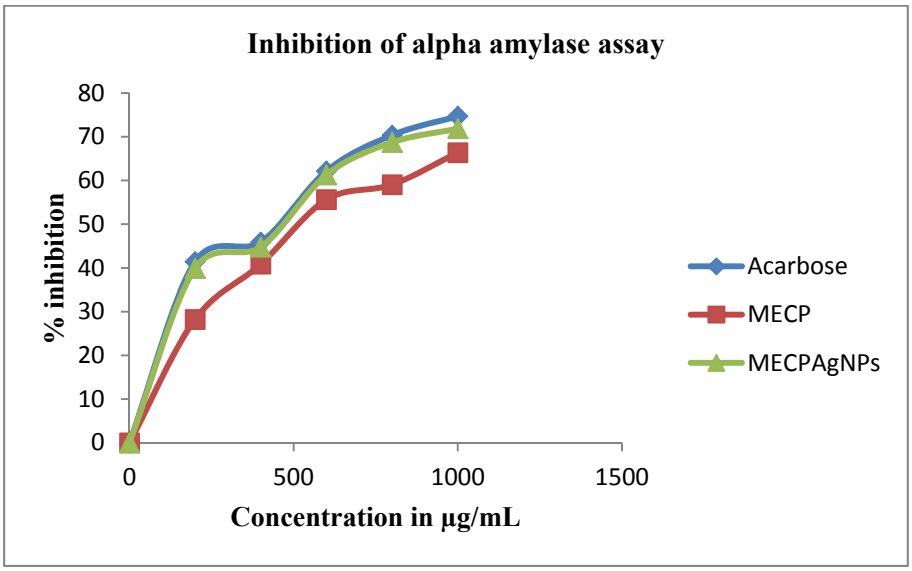
The results obtained for the inhibition of α-amylase enzyme assay are presented in the **Table 22** and the graphical representation in **Fig**. **40.**

Table 22: Inhibition of alpha amylase enzyme assay

Conc. µg/ mL	Acarbose		MECP		MEAgNPs	
	Absorbance at 565nm*	% Inhibition	Absorbance at 565nm*	% Inhibition	Absorbance at 565nm*	% Inhibition
200	0.094±0.001	41.44±1.08	0.07±0.0008	28.23±0.83	0.091±0.001	39.89±0.18
400	0.113±0.003	45.89±1.64	0.094±0.002	40.87±0.45	0.108±0.003	44.77±1.42
600	0.147±0.001	62.14±0.32	0.124±0.001	55.63±0.41	0.142±0.005	61.26±0.16
800	0.184±0.002	70.36±0.26	0.134±0.003	59.05±0.10	0.176±0.005	68.71±1.80
1000	0.224±0.001	74.70±0.58	0.163±0.002	66.37±0.55	0.195±0.002	71.85±0.41
IC_{50}	513.97µg/Ml		639.83 µg/mL		534.39 µg/mL	

*** mean of three readings ±SEM**

Fig. 40: Inhibition of alpha amylase enzyme assay



From the **Table 22,** it can be observed that the MECPAgNPs possess very good antidiabetic activity as compared to the MECP. The *in vitro* α-amylase inhibition study showed that both MECPAgNPs and MECP inhibited α-amylase. The percentage inhibition at the α-amylase concentrations of 200, 400, 600, 800 & 1000 µg/ml by the MECPAgNPs and MECP showed a concentration-dependent reduction. The highest concentration 1000µg/mL of MECPAgNPs, MECP and acarbose showed a maximum inhibition of 71.85±0411, 66.37±0.553 and 74.7±0.582% while the lowest concentration 200µg/mL of MECPAgNPs, MECP and acarbose showed a minimum inhibition of 39.89±0.181, 28.23±0.830 & 41.44±1.076. The MECPAgNPs showed strong α-amylase inhibition as compared with MECP. The IC_{50} values of the MECP, MECPAgNPs and acarbose were found to be 639.83, 534.39 & 513.97µg/mL respectively.

α-amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitors bind to alpha- bond of polysaccharide and prevent break down of polysaccharide in to mono and disaccharide. In our experimental study it was observed that MECPAgNPs demonstrated significant α-amylase inhibition activity as compared to MECP.

METHOD 4: GLUCOSE UPTAKE BY YEAST CELLS

a) INHIBITION OF GLUCOSE UPTAKE IN 5mM GLUCOSE CONCENTRATION

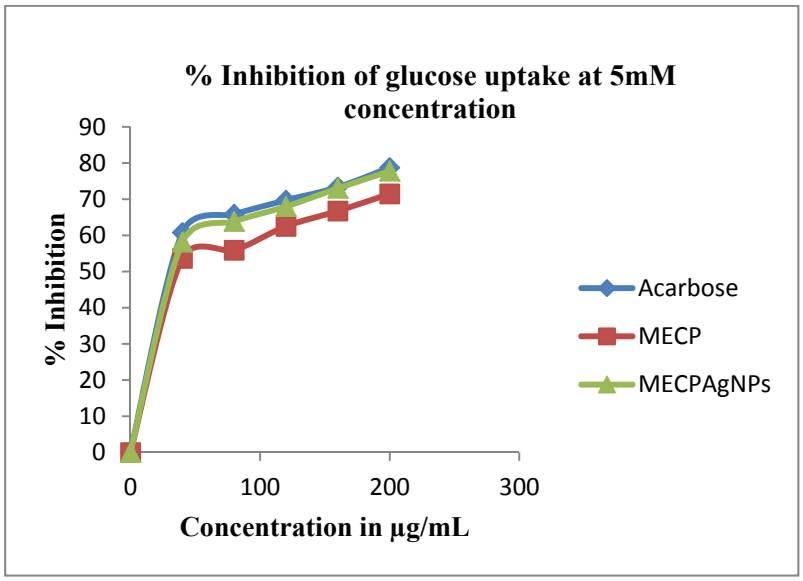
The results obtained for the inhibition of glucose uptake in 5mM & 10mM glucose concentration assay are presented in the **Table 23**. The rate of glucose transport across cell membrane in yeast cells system was explored and the results are given in **Fig. 41.**

Table 23: Inhibition of Glucose uptake in 5mM glucose concentration

Conc. µg/ mL	Acarbose		MECP		MEAgNPs	
	Absorbance at 520nm*	% Inhibition	Absorbance at 520nm*	% Inhibition	Absorbance at 520nm*	% Inhibition
40	0.102±0.001	60.78±0.22	0.072±0.006	53.66±0.36	0.086±0.006	58.17±0.44
80	0.117±0.001	65.81±0.22	0.080±0.008	55.89±0.39	0.096±0.004	63.84±0.18
120	0.132±0.001	69.70±0.27	0.106±0.008	62.49±0.31	0.125±0.002	67.98±0.24
160	0.150±0.002	73.33±0.10	0.120±0.007	66.75±0.24	0.148±0.001	73.01±0.35
200	0.188±0.002	78.72±0.25	0.141±0.001	71.49±0.25	0.186±0.001	77.77±0.43
IC_{50}	74.08 µg/mL		94.09 µg/mL		78.39 µg/Ml	

*** mean of three readings ±SEM**

Fig. 41: Inhibition of glucose uptake at 5mM concentration



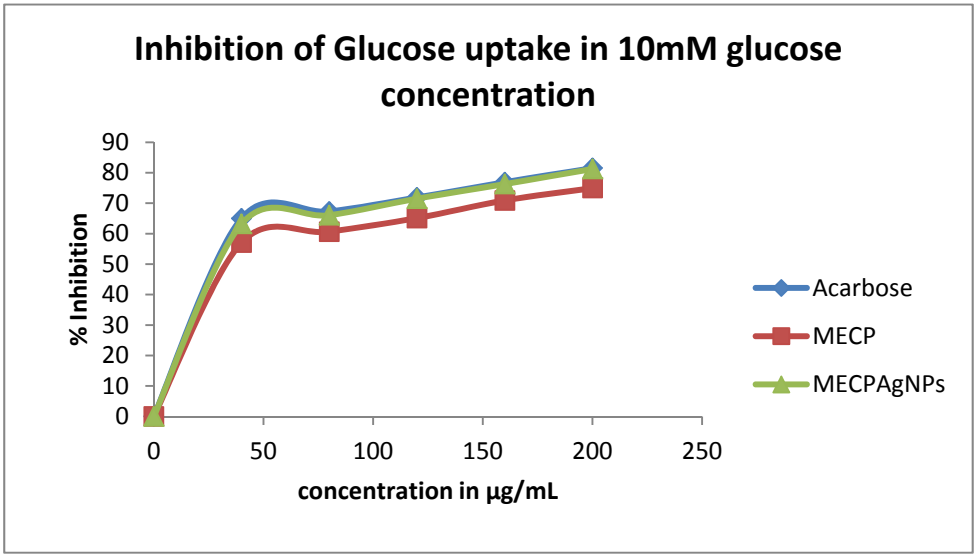
From the **Table 23**, it can be seen that the MECP, MECPAgNPs showed a percentage inhibition of 71.99±0.246 and 77.77±0.430 while acarbose showed a percentage inhibition of 78.82±0.248 at a concentration of 200µg/mL. The IC_{50} value calculated using linear regression analysis was found to be 94.09µg/mL, 78.39 and 74.08µg/mL for MECP, MECPAgNPs & acarbose. The graphical representation of the inhibition of glucose uptake at 5mM glucose concentration is presented in the **Fig. 41.**

Table23. Inhibition of glucose uptake at 10mM concentration

Conc. µg/ mL	Acarbose		MECP		MEAgNPs	
	Absorbance at 520nm*	% Inhibition	Absorbance at 520nm*	% Inhibition	Absorbance at 520nm*	% Inhibition
40	0.200 ± 0.003	65.00±0.24	0.166±0.001	57.07±0.44	0.190±0.008	63.21±0.17
80	0.214 ± 0.002	67.28±0.16	0.198±0.001	60.64±0.18	0.206±0.003	66.05±0.52
120	0.249 ± 0.004	71.88±0.18	0.213±0.001	65.12±0.24	0.229±0.004	71.49±0.55
160	0.303 ± 0.003	76.89±0.21	0.241±0.001	70.91±0.35	0.295±0.003	76.28±0.27
200	0.379 ± 0.001	81.53±0.18	0.267±0.003	74.96±0.79	0.373±0.002	81.24±0.12
IC_{50}	**67.40 µg/mL**		**84.26 µg/mL**		**69.87 µg/mL**	

*** mean of three readings ±SEM**

Fig. 42: Inhibition of glucose uptake at 10mM concentration



From the **Table 23**, it can be seen that the MECP, MECPAgNPs showed a percentage inhibition of 74.96±0.791 and 81.24±0.120 while acarbose showed a percentage inhibition of 81.53±0.181 at a concentration of 200µg/mL. The IC_{50} value calculated using linear regression analysis was found to be 84.26µg/mL, 69.37 µg/mL and 67.40µg/mL for MECP, MECPAgNPs & acarbose respectively. The graphical representation of the Inhibition of Glucose uptake in 10Mm glucose concentration was presented in the **Fig. 42.**

After the treatment of the yeast cells with the MECP, MECPAgNPs the glucose uptake was found to increase in a dose dependent manner. The amount of glucose lingering in the medium after a specific time serves as a marker of the glucose uptake by the yeast cells. The rate of uptake of glucose into yeast cells was linear in the 5mM & 10mM glucose concentrations. Results indicated that MECPAgNPs had greater efficiency in increasing the glucose uptake by yeast cells as compared to MECP in both 5mM & 10mM glucose concentrations

SUMMARY AND CONCLUSION

CHAPTER 9

SUMMARY AND CONCLUSION

The dissertation entitled **"Green synthesis of silver nanoparticles from the methanolic leaf extract of*Costuspictus* D. Don (Costaceae) for enhancing the oral bio-availabilty and its anti-diabetic activity ".**

The chapter on **Review of literature** of the plant *Costuspictus* D. Don (Costaceae) provides information on the pharmacognosy, phytochemical, and pharmacological activities of the various species of *Costus*. It *a*lso reveals the synthesis of silver nanoparticles from various extract of the plants.

The chapter **Pharmacognostical Evaluation** describes the macroscopical, microscopical studies, standardization parameters, quantitative microscopy, cell morphological characters, powder microscopy of the leaves. The following features are observed for this plant.

- It is an erect herb growing up to 3meters tall; the stem is horizontally striped at base. The leaves are narrowly lanceolate, dark green above and lighter green below, small leaves are present on the basal part, bract green, with outer margin coloured maroon. Flowers are yellow, lip with maroon striations, darker yellow stripe down the middle region, anther cream coloured.
- The vascular bundles of the lamina occur in a single horizontal row of the median part of the lamina. The wide circular xylem elements and thick cluster of phloem and parenchymatous bundle sheath.
- The adaxial epidermis is thick and it consisting of narrow tubular cells. The abaxial epidermis is apostomatic. The abaxial epidermis is stomatiferous.

- The stomata are diffuse in distribution. They are surrounded by two pairs of subsidiary cells and one pair of polar subsidiary cells. This type of stomata is called hexacytic stomata.
- Calcium oxalate crystals of minute particle are aggregated in to larger masses in the leaf mesophyll cells.The crystals located in ordinary unspecialized cells.

These characters play an important role in the authentication of crude drug and will also be useful for the detection of adulteration.

- Quantitative analytical microscopy, powder analysis and standardization parameters, evaluation gives values which are constant and will be useful for the identification for its quality and purity, authentication of the crude drug and the plant material.

The chapter **Synthesis of *Costuspictus* D. Don silver nanoparticles** focuses mainly on green route for the synthesis of nanoparticles. For a long time, herbal medicines were not considered for development as novel formulations owing to lack of scientific justification and processing difficulties, such as standardization, extraction and identification of individual drug components in complex polyherbal systems. However, modern phytopharmaceutical research can solve the scientific needs (such as determination of pharmacokinetics, mechanism of action, site of action, accurate dose required etc.) of herbal medicines to be incorporated in novel drug delivery system, such as nanoparticles, microemulsions, matrix systems, solid dispersions, liposomes, solid lipid nanoparticles and so on.

- The biological synthesis of silver nanoparticles using*Costuspictus* extract was shown to be rapid and produced particles of fairly uniform size and shape. As the methanolic leaf extracts of *Costuspictus*D. Don were mixed with the aqueous solution of silver ion complex, it changed into brown colour due to

the excitation of surface Plasmon vibrations, which indicated the formation of MECPAgNPs.

- The nanoparticles were primarily characterized by UV-Visible spectroscopy, which was proved to be very useful technique for the analysis of nanoparticles. In the UV-Visible spectrum, the broadening of the peak indicated the particles are poly dispersed. The surface Plasmon band in the silver nanoparticles in the solution remains close to 420nm. Throughout the reaction period indicating the particles are dispersed in the aqueous solution of silver nitrate, with no evidence for aggregation. The average particle size (z-average) was found to be 132.6nm, its polydispersity index was 0.248 and zeta values were measured and found to -25.1mV with the peak area of 100% intensity. This indicates that the silver nanoparticle formed is stable.
- A SEM images showed that the silver nanoparticles formed were spherical in shape, with an average size of around 100nm. SEM showed uniformly distributed silver nanoparticles on the surface of the cells was observed.

This green chemistry approach towards the synthesis of silver nanoparticles has many advantages such as, ease with which the process can be scaled up, economic viability, etc.

The chapter on **Phytochemical Evaluation** deals with the preliminary phytochemical evaluation and quantitative estimation of phytoconstituents present in the methanolic extract of the plant which gives information on the identify the presence of the secondary metabolites present in*Costuspictus* D. Don. The amount of vitamin C, phenols, tannins and flavonoid content of MECP and MECPAgNPs was compared. These determination and quantification gives the information about the amount of secondary metabolites present in the MECPAgNPs was higher than the MECP which is responsible for the therapeutic or pharmacological activity of the plant.

The chromatographic studies of the plant which includes TLC and HPTLC fingerprint profile was carried out. The MECP was subjected to TLC. The presence of quercetin was confirmed by HPTLC.

The chapter **Pharmacological Evaluation** deals with the comparision of MECPAgNPs and MECP for its *in vitro* anti oxidant and *in vitro* antidiabetic activity.

- The **antioxidant activity** of MECPAgNPs and MECP have exhibited radical scavenging activity by DPPH assay, hydrogen peroxide, FRAB, Reducing power assay, Phosphomolydenum method shown potent anti oxidant activity. The MECPAgNPs showed very potent anti oxidant activity as compared to MECP.
- The *in vitro* **antidiabetic activity** of MECP &MECPAgNPs was screened by 4 methods namely,
    i) Non- enzymatic glycosylation of haemoglobin assay.
    ii) Alpha Glucosidase inhibition assay
    iii) Alpha amylase inhibition assay
    iv) Glucose uptake by Yeast cells

The MECPAgNPs& MECP displayed the inhibition of haemoglobin glycosylation concentration of glucose over the period of 72hrs, indicating that the MECPAgNPs& MECP decreases the formation of the glucose- haemoglobin complex and thus the amount of free haemglobin increases.MECPAgNPs exhibited higher inhibition of glycosylation as compared with the MECP at a concentration of 200-1000µg/mL.

In alpha amylase enzyme inhibition assay and alpha glucosidase enzyme inhibition assay, the results showed MECPAgNPs had efficiently inhibits both alpha amylase, alpha

glucosidase enzyme, ***in vitro*** in dose dependent manner than MECP at a concentration of 200-1000µg/mL.

MECPAgNPs had greater efficiency in increasing the glucose uptake by yeast cells as compared to MECP at a concentration of 200-1000µg/mL.

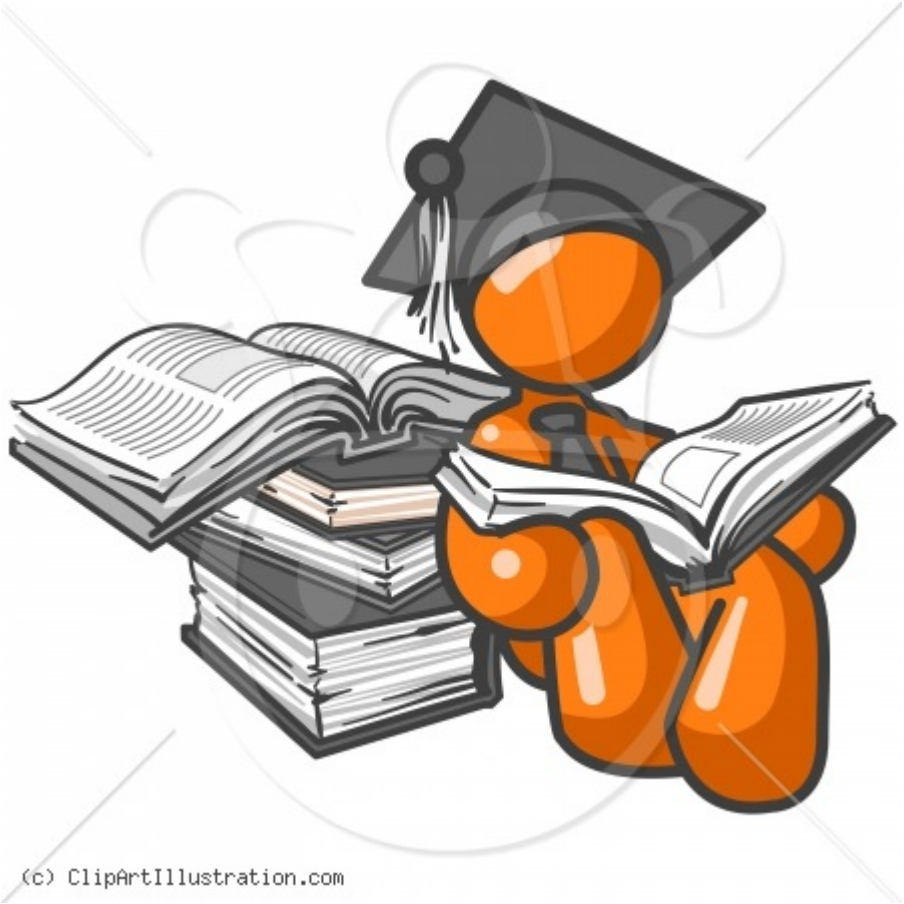
Antidiabetic activity of***Costuspictus*** D. Don might be due to the presence of pentacyclictriterpene compound such as β- amyrin and β- L- Arabinopyranose methyl glucoside in the plant.[78]

Many ***in vivo*** and ***in vitro*** studies of this Insulin plant are carried out showed that this plant is having very potent antidiabetic effect. Though being widely used, no formulation containing this plant is available in the market. The patient feels difficulty in chewing the leaves for a month. In order to overcome this and to enhance the oral bio-availability & pharmacological activity, protection from toxicity, physio-chemical degradation, improved tissue macrophages, to avoid repeated administration of the dose and to achieve the selective or targeted drug delivery towards a specific tissue or organ a attempt is made in this study to synthesis MECPAgNPs.

In an attempt to find natural, environmentally benign, easily available plant- based agents for the synthesis of metal nanoparticles, we have demonstrated the efiiciency of***Costuspictus*** D. Don leaf extract in the rapid synthesis of silver nanoparticles possessing a variety of fascinating morphologies owing to its diverse groups of phytochemicals like phenols, flavonoids, reducing sugars, sterols.

Silver nanoparticles synthesized from MECP showed higher antidiabetic activity as compared to the MECP.MECPAgNPsenhances the dissolution rate by increasing the absorption due to decreasing the particle size (132.6nm) and hence results in increased oral bioavailability.

It can be concluded that the leaves of *Costuspictus* D. Don can be a good source for synthesis of silver nanoparticle which shows potent anti oxidant and antidiabetic activity than the MECP. The important outcome of the study will be the development of value added product from medicinal plant *Costuspictus* D. Don for biomedical and nanotechnology based industries. Overall, thisapproach seems to be one of the best strategies for therapeutic management to treat diabetics.



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